

University of Alberta

**The Differential Apoptotic Effects of Vpr and Vpx
of Human and Simian Immunodeficiency Viruses
in Mammalian Cells**

by

Chih-Hsiung Chen



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in partial fulfillment of the requirements for the degree of
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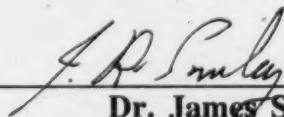
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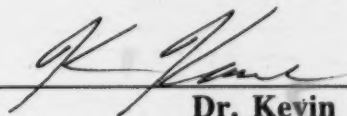
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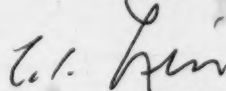
(Supervisor) **Dr. Lung-Ji Chang**



Dr. James Smiley



Dr. Kevin Kane



Dr. C.C. Lin

Date: *November, 20, 1997*

Abstract

Previously published results from our laboratory have shown that HIV-1 Vpr has both cytostatic and cytotoxic effects in the fission yeast Schizosaccharomyces pombe. We transiently transfected different vpr and/or vpx genes from different primate lentiviruses into a variety of mammalian cell lines from different species. These results showed that :

1) HIV-1 Vpr induces apoptosis in human cells (HeLa, TE671 and 293) but not in chimpanzee cells (PT1SK), African green monkey cells (Vero) and rodent cells (CHTG49, A9 and P388D1).

2) In contrast, Vpr and/or Vpx of HIV-2, SIVmac239 and Vpx of SIVagm do not induce apoptosis in any of the cell lines mentioned above.

3) When the HIV-1 vpr gene was cotransfected with a reporter gene, GFP, about 90% of the apoptotic cells were GFP-positive, suggesting that HIV-1 Vpr does not induce apoptosis through paracrine or bystander route.

4) The apoptotic effect of HIV-1 Vpr can be regulated by cotransfecting with different members of Bcl-2 gene family, suggesting that the Bcl-2 family members may be involved in the mechanism of HIV-1 Vpr-induced apoptosis.

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Abbreviations

AIDS: acquired immunodeficiency syndrome.

ATCC: American type culture collection.

BES: N, N- bis(2-hydroxyethyl)-2-aminoethanesulfonic acid.

BSA: bovine serum albumin.

CDC: cell division cycle.

CMV: cytomegalovirus.

CPZ: chimpanzee.

DMEM: Dulbecco's Modified Eagle's Medium.

DTT: dithiothreitol.

ICE: interleukin-1 β converting enzyme.

EBV: Epstein-barr virus.

EDTA: ethylenediamino-tetraacetic acid.

FACS: fluorescence activated cell sorting.

FBS: fetal bovine serum.

GFP: green fluorescence protein.

gP41: glycoprotein 41.

gP120: glycoprotein 120.

HIV-1: human immunodeficiency virus type I.

HIV-2: human immunodeficiency virus type II.

hGH: human growth hormone.

HTLV-1: human T-cell leukemia virus type 1.

Ig: immunoglobulin.

IFN- γ : interferon- γ .

KCl: potassium chloride.

kDa: kiloDalton.

LPS: lipopolysaccharide.

LTR: long terminal repeat.

MA: matrix protein.

MEM: minimal essential medium.

NF- κ B: nuclear factor κ B.

NLS: nuclear localization signal.

NP-40: Nonidet P40.

PAGE: polyacrylamide gel electrophoresis.

PARP: poly(ADP-ribose) polymerase.

PCR: polymerase chain reaction.

PI: Propidium iodide.

PMA: phorbol-12-myristate-13-acetate.

PMSF: phenylmethylsulfonyl fluoride.

PP2A: protein phosphatase 2A.

RIA: radioimmunoassay.

PBS: phosphate buffered saline.

Rip-1: Vpr interaction protein-1.

RNase A: ribonuclease.

SIV: simian immunodeficiency virus.

TAR: transactivation response element.

TAK: Tat-associated protein kinase.

TBP: TATA box binding protein.

TGF- β 1: transforming growth factor β 1.

TdT: deoxynucleotidyl transferase.

tetOP: tetracycline operon.

TFIID: transcription factor IID.

TNF- α : tumor necrosis factor- α .

TRE: Tetracycline response element.

UNG: uracil DNA glycosylase.

VSV: vesicular stomatitis virus.

Vpr: Viral protein R.

Vpx: Viral protein X.

Chapter I.

Introduction

The primate lentivirus family.

The lentivirus is one of the subclasses of the retrovirus family. A variety of mammals such as domestic cats, cattle, horses, goats and sheep and primate species can be infected naturally by lentiviruses (1-7). Recently, the primate lentiviruses have attracted attention because they have been identified as the etiologic agents of human acquired immunodeficiency syndrome (AIDS) and simian AIDS (8,9). Five phylogenetic lineages in primate lentiviruses have been identified: HIV-1/SIVcpz, HIV-2/SIVsm/SIVmac, SIVagm, SIVmnd and SIVsyk (Figure 1) (10-14). Human immunodeficiency virus type 1 and 2 (HIV-1 and HIV-2) have been isolated from AIDS patients and identified as the etiologic agents for AIDS in humans. Simian immunodeficiency virus originally isolated from some African monkeys including sooty mangabeys (SIVsm) (15,16), African green monkeys (SIVagm) (17), mandrills (SIVmnd) (18), sykes monkeys (SIVsyk) (19), and chimpanzees (SIVcpz) (20) can not cause any virus-related disease in their natural hosts. Instead, the viruses cause a persistent asymptomatic infection. However, when Asian macaques (e.g. rhesus, cynomologus, stumptailed and pigtailed macaques) are experimentally infected with the SIV strains mentioned above, they may develop an acquired immunodeficiency syndrome resembling AIDS in humans (21-24). Thus, the primate lentivirus in Asian macaques is a very valuable animal model to the research of diseases associated with human acquired immunodeficiency syndrome.

The viral genomes of primate lentiviruses

The lentivirus genomes possess three genes (gag, pol, env) that are also found in the genomes of other retrovirus members. However, the lentivirus genomes also contain at least six other accessory genes called tat, rev, vif, vpr, vpu and nef which can only be found in primate lentiviruses (Figure 2) (25). Two of the accessory genes, tat and rev, are required for virus replication (26-28). The tat gene product is a transactivation factor for the proviral long terminal repeat (LTR), and the rev gene product is required to ensure the proper shifting from the early to the late phase of viral gene expression. The gene products, vif, vpr, vpu, nef and vpx are not essential for virus replication in some culture systems *in vitro*; therefore, these proteins are often called non-essential accessory proteins for viral replication. However, the high homology found among these non-essential accessory factors of different primate lentiviruses indicates that they may have very crucial functions for virus replication and pathogenesis *in vivo*.

The cytopathic effects in acutely and chronically viral-infected cells and HIV-1 pathogenesis.

AIDS is characterized by a progressive depletion of CD4-positive cells (e.g. lymphocytes, monocytes and dendritic cells) and eventually an immunocompromise in responding to the challenges from a variety of foreign microorganisms (29,30). The way that HIV causes the decline of numbers of CD4-positive cells in the immune system is still not very clear. In an acute infection, HIV-1 causes some cytopathic effects in CD4-positive lymphocytes such as syncytium formation (multinucleated giant cells) or single-cell lysis (31,32). Syncytium formation is mainly mediated by HIV-1 envelope glycoproteins, gp120 and gp41 which are expressed on the surface of virus particles (33-37).

Apoptosis and HIV/SIV pathogenesis

Apoptosis, is one of the mechanisms of cell death caused by HIV-1 infection. Apoptosis, or called " **Programmed Cell Death** ", is a morphologically well-defined process (38). It is distinguished from necrosis by characteristic morphological alternations, e.g. cytoplasmic contraction, chromatin condensation, and DNA cleavage into oligonucleosomal fragments. Apoptosis is essential for the proper control of embryonic development in vertebrate to maintain homeostasis. In addition, apoptosis also acts as a way to remove abnormal cells which are induced by oncogenic transformation and viral infection (39). Normally, there are balances between cell proliferation and apoptosis. It has been reported that a variety of diseases such as cancer and autoimmune diseases are related to the abnormal blocking of apoptosis and that results in cell proliferation without control (126,127). In contrast, if apoptosis is enhanced too much, it will cause diseases such as neurodegenerative diseases and AIDS (128).

Until recently, it has been hypothesized that the induction of apoptosis in HIV-1-infected or non-infected cells in vivo could be the main reason for the depletion of CD4-positive lymphocytes in AIDS patients (40). The studies showed that a high level of apoptosis was found in SIV-infected macaques and in HIV-1-infected humans who both could develop AIDS but not in HIV-1- infected chimpanzees which could not develop AIDS (41-43). In addition, the levels of apoptosis induction in CD4-positive T cells were correlated to the disease progression in HIV-1 infected humans and SIV-infected macaques.

The basic machinery of apoptosis in mammalian cells

The network of apoptosis is quiet complicated. Different stimuli may induce different signaling pathways (44). Nevertheless, the process of apoptosis can be divided into three

different phases: initiation, effector, and degradation (or executioner) phases according to the types of stimulation, time, and cellular location (45). The initiation stage depends on a variety of apoptotic stimuli which determine the induction pathway. For instance, it is believed that the apoptosis signaling induced by glucocorticoids is different from that induced by Fas/CD95/TNF- α in the initiation stage because Bcl-2 can efficiently inhibit the apoptosis induced by glucocorticoids but not by Fas/CD95/TNF- α (46,47). The signals coming from the initiation stage are transferred to the regulation effectors such as the Bcl-2 family members and then enter the degradation stage.

Recent studies have shown that the regulation of mitochondria permeability transition by the Bcl-2 family is a central effector in the apoptotic events (48,49). When apoptosis signaling is initiated, there is a reduction in the mitochondria transmembrane potential due to the opening of the mitochondria permeability transition pores. Some executioner factors, such as cytochrome c or AIF (apoptosis-initiating factor) will be released from the mitochondria into the cytosol (50-52). In addition, the execution factors will trigger more executioners (e.g. cytochrome c can activate Yama/CPP32, a member of the ICE-like protease family) and eventually induce the irreversible apoptosis signaling cascades related to the ICE-like protease family. It has been reported that overexpression of Bcl-2 can prevent mitochondria permeability transition and further inhibit the release of cytochrome c into the cytosol (52). In addition, Bcl-X_L, another Bcl-2 family member is able to prevent apoptosis and forms cation channels in the mitochondrial outer-membrane, suggesting that Bcl-X_L also prevents the mitochondria permeability transition (53).

The Ced-3/ICE-like protease family

Most of our knowledge about the family of ICE-like protease comes from the studies of the developmental cell death of *Caenorhabditis elegans*. There are three ced genes that organize cell death (54). Ced-3 and ced-4 genes enhance apoptosis of target cells whereas

ced-9 acts to block the killing function of ced-3 and ced-4. Ced-9 is a structural and biological homologue of Bcl-2 in vertebrates. There are no functional homologues of ced-4 found in vertebrates. Ced-3 encodes a cysteine protease related to an interleukin-1 β -converting enzyme (ICE) in vertebrates (55). There are nine members in the ICE-like protease family which include the Ced-3-like subfamily members (CPP32 also known as Yama/Apopain, Mch2, Mch3 also known as ICE-LAP-3, and Mch4); the ICE subfamily members (ICE, ICE rel III, and ICE rel II also known as TX/ICH2); and the NEDD-2 subfamily members (ICH-1 and Nedd-2). Recently, the ICE, CPP32, and Mch2 have been implicated to be involved in the signaling cascades of apoptosis (55).

The control of cell death by the apoptosis enhancers ced-4 or ced-3/ICE-like proteases, and the apoptosis suppressers, ced-9/Bcl-2.

The individual aspects of ced-9/Bcl-2 and ced-4/ced-3 have been well identified in apoptosis signaling. However, the link between ced-9/Bcl-2 and ced-4/ced-3 is still not very clear (56). Recently, it was reported that overexpression of ced-4 in mammalian cells can induce apoptosis, but the induction can be blocked by overexpression of Bcl-X_L and ced-9 (57). In addition, direct physical interactions between ced-4/Bcl-X_L, ced-4/ced-9, and ced-4/ced-3 have been demonstrated by immunoprecipitation (58).

The ced-9 and Bcl-X_L proteins are localized primarily in the intracellular membranes and the perinuclear regions as is Bcl-2. In contrast, ced-4 and ced-3 are localized in the cytosol (58). Ced-9/Bcl-X_L and Bcl-2 can target ced-4 from the cytosol to the intracellular membranes, preventing the binding of ced-4 to ced-3, inhibiting the function of ced-3 and other ICE-like proteases, and blocking the apoptosis signaling cascades in mammalian cells (58).

The possible targets of ICE-like protease family members.

The ced-3/ICE-like protease family functions as the basic executioners in the apoptosis signaling cascades through the proteolysis of specific targets. The cleavage of the cellular targets such as the nuclear enzymes poly(ADP-ribose) polymerase and DNA-dependent protein kinase both involved in the recognition and the repair of DNA damage, the U1 ribonucleoprotein, the nuclear lamins, and some components of the cytoskeleton (e.g. actin) may cause the characteristics of apoptosis such as DNA fragmentation, chromatin condensation, and cell membrane blebbing (55).

Vpr, Vpx, and their suspected roles in HIV/SIV pathogenesis.

The vpr gene is found in HIV-1/SIVcpz, HIV-2/SIVsm/SIVmac, SIVmnd and SIVsyk. There is an additional gene in the genomes of HIV-2/SIVsm/SIVmac called vpx. It is suggested that vpr and vpx are duplications from the same ancestors (59). SIVagm does not have the vpr gene but has the vpx, which is highly homologous to the vpr of HIV-1/SIVcpz and the vpr and vpx of HIV-2/SIVsm/SIVmac (59).

Vpr, also known as viral protein R, is believed to be another possible cause of the depletion of CD4-positive-T cells in AIDS patients (60). Vpr is a 14 kDa protein with 96 amino acids. The vpr gene overlaps with the vif gene at its 5' end and with the tat gene at its 3' end. The conservation of the vpr open reading frame in the evolution of the primate lentiviruses indicates that the vpr gene is crucial for viral function (59). It has been shown that vpr is dispensable for viral replication in T cell lines and in primary activated peripheral blood mononuclear cells (61) but is necessary for the viral replication in resting T cells or terminally differentiated macrophages (62).

In addition, it has been shown that Vpr is a weak transactivation factor to the HIV long terminal repeat (LTR) and several other viral promoters such as human T lymphotropic virus 1, cytomegalovirus, and Epstein-Barr virus (60). It also has been reported that the vpr gene product is packaged into viral particles by binding to the carboxyl terminus of Gag precursor p6 (63-66). It is indicated that Vpr may function as an activator of viral transcription in the early phase of the virus life cycle in a tat-independent manner (67,68).

The nuclear localization roles of Vpr and MA proteins.

The reason that HIV can infect non-dividing cells such as terminally differentiated macrophages or resting T cells is that the HIV genome preintegration complex can enter the nucleus of the cells during interphase (69). That is a very distinct difference between lentiviruses and other retroviruses. The matrix protein (MA) and the Vpr protein are included in the HIV preintegration complex and appear to be involved in two distinct mechanisms for the entry of the HIV genome into the nucleus: An importin-dependent pathway is mediated by MA (70,71) while the importin-independent pathway is mediated by Vpr (72,73). Viruses with either these two genes deleted can still infect macrophages (74), suggesting that these two proteins act in a redundant manner. MA protein has highly conserved basic residues in the amino terminus which serve as a nuclear localization signal (NLS) (75). During virus particle packaging, MA protein is phosphorylated at the C-terminal tyrosine site, and binds to the viral integrase (IN) to become a part of the viral preintegration complex. When viruses infect target cells, the preintegration complex is transferred into the nucleus through the binding of MA proteins and the karyopherin $\alpha\beta$ heterodimers (76).

In contrast, it is still not clear how Vpr protein helps the viral preintegration complex enter the nucleus because Vpr protein does not have a typical NLS (77). It has been reported that

there are two regions in the amino terminus of vpr which form amphipathic helices and they seem to be crucial to keep Vpr protein in the nucleus (78).

A variety of cellular factors have been found to bind to Vpr protein. For instance, the transactivator factor Sp1 (79) and a 41 kDa cytosolic protein Rip-1 (Vpr interacting protein-1) along with the glucocorticoid receptor (80). It has been shown that the Rip-1 protein and the glucocorticoid receptor can be coprecipitated with Vpr protein by immunoprecipitation. It is indicated that Vpr protein may translocate the virus preintegration complex into the nucleus by either itself or binding to other cellular proteins (80).

The cytostatic and cytotoxic effects of Vpr protein.

Recently, the HIV-1 Vpr has been shown to have cytostatic effect in both mammalian cell (81-84) and fission yeast cell systems (85). Analysis of the inhibition effects of Vpr in cell growth by FACS shows an increase in the ratio of the cell populations in the G2/M phase to those in the G1 phase in those cells expressing vpr genes (81). It is suggested that the HIV-1 Vpr-induced cell cycle arrest at the G2 phase is due to the inhibition of p34/CDC2 kinase activity (86-88). HIV-2 and SIV Vpr have also been reported to induce cell cycle arrest but the potencies are not as strong as that of HIV-1 Vpr. In addition, the Vpx protein from HIV-2 and SIVmac239 does not induce cell cycle arrest (89). However, the Vpr and Vpx proteins both have cytostatic effect in inducing cell cycle arrest at the G2/M phase and cytotoxic effect in the fission yeast Schizosaccharomyces pombe (9).

Purpose of this study

The results of previous studies have shown that HIV-1 Vpr has cytostatic but not cytotoxic effects in human T cell lines (87). However, studies published from our

laboratory have shown that HIV-1 Vpr has both cytostatic and cytotoxic effects in fission yeast Schizosaccharomyces pombe. (9). In this study, we extended the investigation from the yeast cells to different mammalian cell lines and tried to investigate the possibility that the vpr and/or vpx gene products from different primate lentiviruses such as HIV-1, HIV-2, SIVmac239 and SIVagm have cytotoxic effects in mammalian cells, the possible mechanisms, and their contribution to the HIV/SIV pathogenesis.

Chapter II.

Materials and Methods

Cells and culture conditions:

HeLa cells (human cervix carcinoma, obtained from the American Type Culture Collection, ATCC, Rockville Maryland), TE671 cells (human rhabdomyosarcoma, obtained from ATCC), 293 cells (transformed human primary embryonic kidney cells, obtained from ATCC), PT1SK cells (chimpanzee abnormal skin cells, obtained from ATCC), CHTG49 cells (Chinese hamster ovary cells) and A9 cells (mouse connective cells) obtained from W. McBride and C. Kozak respectively, were propagated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco Canada). P388D1 cells (mouse monocyte-macrophage cells) were maintained in RPMI 1640 (Gibco) with 15% FBS. Vero cells (African green monkey kidney cells, obtained from ATCC) were maintained in minimal essential medium (MEM, from Gibco) supplemented with 5% FBS. All of the growth media were supplemented with 5 ml L-glutamine (Gibco) and 5 ml penicillin-streptomycin per 500 ml growth medium.

The plasmid construction:

The vpr, vpx and other viral genes from different members of the lentivirus family (obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) were amplified by PCR. The PCR products were ligated into a pNREP tet-

off system (made by Dr. L-J Chang) or a pHEF1 α eukaryotic cell expression vector. The primers used to make PCR products are listed in Table 1 and have been described previously (9).

PCR products generated as described above were treated with T4 DNA polymerase blunt-end ligation and subcloned into a pKS-BS(-) vector (Stratagene) previously digested with EcoR V. The PCR fragments of HIV-1 Vpr, HIV-2 Vpr, Vpx, SIVmac239 Vpr, Vpx were then excised by digesting with EcoRI/BamHI and ligated into a pHEF1 α vector previously digested with EcoRI/BamHI. In order to construct the pHEF-SIVagm vpx, the PCR products of SIVagm vpx were subcloned into pKS-BS(-), digested with XbaI/KpnI, and then ligated with pHEF-nef plasmid (from Dr. L-J Chang) previously digested with XbaI/KpnI.

The pHEF-GFP plasmid was provided by Dr. L-J Chang and the pcDNA-Bcl-2, -BAX, -Bcl-XL and -Bcl-Xs were provided by S. N. Farrow, United Kingdom (UK).

The PCR fragments of vesicular stomatitis virus envelope VSV-G gene, obtained from T. Hobman (University of Alberta, Edmonton), HIV-1 Vpr and IFN- γ were ligated into a pNREP-tetOP-hGH vector (by Dr. L-J Chang). The pNREP-tetOP-hGH (or called tet-hGH) vector was made by Dr. L-J Chang constructed as follows: the pNREP vector originally was made by digesting a pNREP9 vector (Invitrogen, CA) with XbaI/AvrII to remove a fragment containing the EBNA-1 and the oriP sequences of EBV from pNREP9 vector. Next, the tet-OP fragment from a pUHD 10-3 plasmid (Display Systems Biotechnology, Inc.), the human growth hormone gene from a XGH5 plasmid (Nichols Institute Diagnostics), and the poliovirus internal ribosomal entry site (P-IRES, obtained from N. Sonenberg) were ligated with pNPEP vector to make the pNREP-tetOP-hGH vector. The pNREP-tetOP-hGH vector has a promoter (tetOP) from the tetracycline-resistance gene operon including 7 repeats of the tetracycline response elements (TRE), as

well as a human growth hormone gene beside tetOP as an internal reporter gene. In addition, the pNREP-tetOP-hGH expression vector requires to be cotransfected with another plasmid UHD-15-1 (Display Systems Biotechnology, Inc.) in order to induce the expression of the genes in the pNREP-tetOP-hGH vector. UHD 15-1 plasmid expresses a fusion protein tetR-VP16 (also called tTA protein) which is composed of a tet repressor (tetR) along with the activation domain of VP16 of herpes simplex virus. tTA protein can bind to tetOP and induce the expression of genes located in the downstream of tetOP (e.g. the human growth hormone gene).

Mammalian cell transfections:

The transfection of mammalian cells was done by either the calcium chloride precipitation method or electroporation. Mammalian cells such as HeLa, TE671, 293, Vero, PT1SK, CHTG49, and A9 were transfected by the calcium chloride precipitation method (90). Briefly, cells were split into a 6-well plate 20 hr before transfection at a confluence of 80%. 5 µg of plasmid DNA in 90 µl of sterile ddH₂O (Gibco Canada) was mixed with 10 µl of 2.5 M CaCl₂ in a polycarbonate tube (Becton Dickinson). In addition, 100 µl of 2X BES buffer solution (50 mM N, N- bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, 280 mM NaCl, 1.5 mM Na₂HPO₄, pH 6.95) was slowly added to the tubes. This DNA solution was incubated at room temperature for 30 to 45 min before it was added to the culture medium. The culture medium was replaced with the fresh DMEM (Gibco) containing 10% FBS before adding the DNA solution into the cultures. After the DNA solution was added, the cultures were maintained in a 3% CO₂ incubator at 37°C for 24 hr. 24 hr after transfection, the culture medium was changed, the CO₂ concentration of the incubator was changed to 5%. 48 hr after transfection, the cells were harvested and the amount of human growth hormone in the medium was measured as described below for the transfection efficiency.

The transfection of P388D1 cells was done by electroporation method (91). Briefly, cells were grown to 90% confluence in a T-75 flask (75 cm²), harvested by using a rubber policeman, washed once with complete culture medium, resuspended in 400 μ l complete culture medium and adjusted to a concentration of 2.5×10^7 /ml. The cell solution was transferred to a 1 ml sterile electroporation cuvette containing 20 μ g of DNA and 3 μ g of pHEF-GFP plasmids as an internal reporter plasmid. After the cell solution and DNA were mixed by tapping, the electroporation was done in an electroporator (Bio-Rad Gene, Inc) at 300 V, 250 μ F. After electroporation, a cuvette containing the cell solution was kept on ice for 5 min. The cells were resuspended in 1 ml complete culture medium, transferred to a T-25 (25cm²) flask, incubated at 37°C overnight, and the culture medium was changed in the next morning. The cells were harvested 48 hr after transfection, stained with Hoechst dye and checked for the percentage of apoptosis.

The transfection efficiency assay:

To determine the transfection efficiency, the amounts of human growth hormone in the medium were measured by using a commercial HGH-TGES transient gene expression kit (Nicols Institute Diagnostics). Briefly, 50 μ l of culture medium was taken from each sample and put into a 2 ml eppendorf tube. Next, 50 μ l of ¹²⁵I-antibody solution was added to each tube followed by adding an avidin-coated bead. Each eppendorf tube was put into a 37°C shaker and shook for 1 hr and then washed three times with washing solution. Subsequently, the beads were put into glass scintillation counter vials, filled with scintillation fluid and the cpm was determined by a scintillation counter (LS 6000TA, Beckman USA). The numbers of cpm were used to normalize the percentage of apoptosis from different samples. If the differences of cpm from different samples were within $\pm 10\%$, the transfection efficiency of each sample was considered the same and the normalization of the percentage of apoptosis from different samples could be ignored.

The cell viability assay: MTT assay/Trypan blue exclusion method.

In the trypan blue exclusion method, cells were harvested at each given time and 0.1 ml cell solution was mixed with the same volume of 0.4% (w/v) trypan blue stain (Gibco). The viability of cells were calculated by counting the percentage of trypan blue-exclusive cells in the total cell population. In the MTT assay, 200 μ l cell solution at 50,000/ml concentration in growth medium was transferred into a 96-well plate, mixed with 20 μ l MTT solution (5 mg/ml 3-(4,5-Dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide; Thiazolyl blue, Sigma), and incubated at 37 °C for 4 hr. Next, the 96-well plate was centrifuged at 1,000 rpm for 5 min (GLC-1 centrifuge, Sorvall) and the supernants were removed from the plate. 100 μ l 0.1 N HCl-saturated isopropanol was added into each well to dissolve the purple-colored particles in the solution. Subsequently, the optical density (O.D.) of each sample in the plate was measured in an ELISA plate reader using the wavelength L1/L2: 570nm/650nm. If the viability of non-treated cells is considered as 100%, the viability of cells treated under different situation is calculated by the ratios of the O.D.(treated cells) to the O.D.(non-treated cells).

The Analysis of Apoptosis

Hoechst staining.

Briefly, cells were harvested from a 6-well plate 48 hr after transfection, pelleted at 4°C and 300 x g for 10 min and washed once with PBS (92). The cells were resuspended in 50 μ l of 4% paraformaldehyde in PBS, incubated for 10 min at room temperature, washed with PBS once and resuspended in 10 μ l of PBS containing 32 μ g/ml of bis-benzimide (Hoechst 33258, Sigma). After a 15 min incubation at room temperature, cells were scored under a fluorescence microscope (BHS-RFL-LSM, Olympus, Tokyo, Japan) with a BH2-

DMU2UV Dich mirror cube filter for the percentage of apoptotic cells. Only cells with three or more chromatin fragments (bright spots) are considered as apoptotic cells. In order to increase the cell density for taking photographs, cells were plated on coverslips. After Hoechst staining, the coverslips were checked under a fluorescence microscope (Axioskop ZEISS, West German) with a combination filter consisting of a blue DAPI fluorescence filter (for the Hoechst stain) and a green FITC fluorescence filter (for the GFP protein). Quantitative analysis was performed by counting more than 500 cells in each examination.

Tunel staining.

Briefly, cells were harvested from a 6-well plate 48 hr after transfection, pelleted at 4°C 300 x g for 10 min, and washed with PBS-1% BSA solution once (93). Cells were pelleted, resuspended in 4% paraformaldehyde solution and incubated on ice for 1 hr. After washing with 1X cold PBS-1%BSA, cells were spun down and resuspended again in remaining supernatant and 100 µl of ice-cold 70% ethanol was added dropwise. The cells were gently resuspended in ethanol solution and kept at -20°C for at least 1 hr. The cells were washed with 1X PBS-1%BSA, pelleted and resuspended in 50 µl reaction solution (1X TdT buffer (Promega), 0.01 µM biotin-labeled dUTP (B.M.) and 10 units terminal deoxynucleotidyl transferase (TdT) in sterile MQ H₂O (Gibco) and incubated at 37°C for 1 hr. After washing with 1X PBS-1% BSA, cells were resuspended in 100 µl staining buffer containing 2.5 µg/ml avidin-Neutralite fluorescein and kept in dark at room temperature for 30 min. Subsequently, the cells were washed twice with 1X PBS-1% Triton X-100, spun down, resuspended in 0.5 µl PBS, and analyzed by FACS analysis (Becton Dickinson).

Propidium iodide staining.

Briefly, cells were harvested as described above, washed with 1X cold PBS, spun down, and resuspended in 2 ml PBS-FBS (1:1 (v/v)) solution (94). The cells were fixed in 6 ml of ice-cold 70% ethanol added dropwise, mixed constantly and incubated at 4°C for at least 30 min. After washing with 1X PBS once, the cells were resuspended in 1 ml of 50 µg/ml Propidium iodide (Sigma) in 1X PBS with 10 µl of 100 mg/ml RNase A (Sigma) and transferred to a polystyrene tube (Falcon). The cell solution was incubated for 20 min on ice, protected from light, and then analyzed by FACS analysis (Becton Dickinson).

The detection of bystander apoptotic effect by the ³H-thymidine release assay.

Briefly, TE671 cells were split into a 24-well plate 24 hr before being labeled. The ³H-thymidine (1 mCi/ml, NEN, Dupont USA) was diluted (1:400) in complete DMEM supplemented with 10% FBS and 0.5 ml medium solution including ³H-thymidine was added to each well for 16 hr. Next, cells were washed three times with complete DMEM in order to get rid of the ³H-thymidine outside the cells. The HIV-1 Vpr, hIFN-γ, negative control HIV-1 Nef-transfected cells, or 100 µg/ml cycloheximide was added on top of the ³H-thymidine-labeled cells. At 24, 36, 48, 60, and 72 hr after coculturing with transfected cells or 24, and 48 hr after cycloheximide treatment, 200 µl supernants were harvested, transferred to the glass scintillation counter vials, filled with 2 ml scintillation fluid and the values of cpm were determined by a scintillation counter (LS 6000TA, Beckman USA). The percentage of apoptosis was calculated by the ratios of the amounts of ³H-thymidine in the supernants subtracted the amounts of ³H-thymidine spontaneously released to the total amounts of ³H-thymidine absorbed by the cells.

Chapter III.

Results

The construction of different gene expression vectors, the transfection systems, and the apoptosis analysis.

The genes coding for different proteins are cloned into the following two eukaryotic cell expression vectors: the tet-off system including a pNREP-tetOP expression vector and a UHD 15-1 vector (Figure 3A), and the pHEF 1 α expression vector (Figure 3B). The pNREP-tetOP vector has a promoter tetOP, which includes the tetracycline response element, and needs to be cotransfected with another plasmid UHD 15-1 in order to induce the expression of the genes in the pNREP-tetOP vectors. UHD 15-1 plasmid, under the control of a CMV promoter, expresses a fusion protein tetR-Vp16AD (tTA protein) consisting of the tetracycline repressor protein (tetR) and the activation domain of VP16 of herpes simplex virus. When tetracycline is withdrawn from medium, tTA protein binds to the tetOP site in the pNREP-tetOP vector and induces the expression of the genes. However, when tetracycline exists in medium, it binds to tTA protein, blocks the binding of tTA protein and tetOP, and inhibits the expression of the genes in the pNREP vector. The pNREP inducible vector is bicistronic, which includes a human growth hormone gene (hGH) in the first cistron as an internal reporter gene for measuring the transfection efficiency. The second cistron is occupied by the genes relevant to this study (e.g. HIV-1 Vpr, positive control hIFN- γ or negative control VSV-G). The pHEF1 α vector was used for the cloning of HIV-1 Vpr, Nef, HIV-2, SIVmac239 Vpr, Vpx, SIVagm Vpx, hIFN- γ , and GFP genes.

Originally, we tried to establish a stable transfection system in HeLa or TE671 cells where the expression of vpr was inducible by using the tet-off system (by Dr. L-J Chang and Jergen Chu). The induction level of hGH in the culture supernatant was measured by a radioimmuno assay (RIA). The establishment of the Vpr-stable expression cell lines was unsuccessful and possible due to the leaky expression of vpr resulted in cytostatic effects in the cells (personal communication from Dr. L-J Chang). Thus, we developed a transient transfection system by using pNREP-tetOP or pHEF 1 α constructs for the Vpr study. In the pHEF 1 α plasmid system, it needs to be cotransfected with 0.1 μ g human growth hormone plasmid XGH5 as an internal control in order to measure the transfection efficiency of each sample. However, it is not necessary in the pNREP-tetOP plasmid system because the human growth hormone gene had been included.

pNREP-tetOP plasmid system mainly was used in the study of the effect of HIV-1 Vpr in different mammalian cell lines from various species. Because we found that tetracycline did not have significant effects on the regulation of gene expression in our tet-off transient transfection system, all transfections with pNREP-hGH-derived constructs were done without tetracycline in culture medium. pHEF-1 α plasmid system mainly was used to compare the differences in the apoptotic effects of the Vpr and/or Vpx gene of various lentivirus family members in different mammalian cell lines from various species.

The plasmid DNA was transfected into cultured cells by using calcium chloride phosphate precipitation method. At 24, 36, 48 hr after transfection, cells were harvested, checked with the viability by MTT assay, or fixed with 4% paraformaldehyde, stained with Hoechst dye as described in the Materials and Methods, and the percentage of apoptotic cells was counted under a fluorescence microscope by examining the morphological changes of apoptotic cells.

Apoptotic cell has very typical morphological changes such as DNA condensation, fragmentation, and cell membrane blebbing which were used to distinguish apoptotic cells from normal cells or even dead cells killed by other mechanism such as necrosis. The morphological difference between normal and apoptotic cells checked by Hoechst stain is shown in figure 4. 100 $\mu\text{g/ml}$ of cycloheximide was used to induce apoptosis in HeLa cells. 48 hr after treatment, cells were harvested, fixed with 4% paraformaldehyde, stained with Hoechst dye, and checked under a fluorescence microscope as described in the Materials and Methods. The morphology of nucleus in normal cells is an intact-round shape (closed arrow). However, the morphology of nucleus in apoptotic cells becomes a few condensed-round spots (open arrow). Only cells with three or more chromatin fragments (bright spots) are considered as apoptotic cells.

In addition, we used other methods such as Tunel and PI stain to analyze apoptotic cells and compared the results by different methods. To avoid personal bias, these experiments were done blinded especially in the visual counting of apoptotic cells by Hoechst stain. First, we used 100 $\mu\text{g/ml}$ cycloheximide to induce apoptosis in HeLa cells and the results shown by different methods were similar (Table 2). Next, we checked the percentage of apoptosis induced by HIV-1 Vpr in HeLa cells by different methods. Cells were transfected with 5 μg of the tet-hGH-derived HIV-1 Vpr, VSV-G constructs or empty vector and the tet-hGH-VSV-G or empty vector was used as negative control. The background values of apoptosis determined by Tunel and PI stain were much higher than those determined by Hoechst stain in our transient transfection system (Table 2) and the reasons we did not know. So we chose the Hoechst stain as our standard apoptosis assay method.

The MTT assay is a better method than the trypan blue exclusion method for determining the viability of cells in transient transfection system.

The MTT assay and trypan blue exclusion method are two popular methods in the study of cell viability. Before we decided which methods could be used to determine the viability of the cells in our transient transfection system, we compared the difference in the viability of TE671 cells transfected with HIV-1 Vpr, hIFN- γ , or empty vector at 24, 36, and 48 hr after transfection by these two methods. The results are illustrated in Table 3. It is shown that the viability of cells determined by trypan blue exclusion method is significantly lower than that determined by MTT assay at 24 hr after transfection. However, there are no significant differences between the results by these two methods at 36 and 48 hr after transfection. According to the procedure of transfection, the transfection solution would not be replaced to fresh culture medium until 24 hr after transfection. It is possible that the transfection solution may influence the accuracy of the trypan blue exclusion method especially in a short period of time after transfection. So we chose the MTT assay as our standard cell viability method.

The cytotoxicity induced by HIV-1 Vpr and hIFN- γ in different mammalian cell lines.

Since HIV-1 Vpr has been shown to have cytotoxicity in fission yeast from the studies published before in our laboratory (9), we extended our investigation from yeast cells to mammalian cells to see if HIV-1 Vpr also has cytotoxicity in mammalian cells. Three human cell lines (HeLa, TE671, and 293) were transfected with HIV-1 Vpr construct in a pNREP-tetOP (tet-hGH) vector. The tet-hGH-VSV-G or the empty vector was as negative control and the tet-hGH-hIFN- γ was as positive control. At 24, 36, and 48 hr (or 24, 48, and 72 hr in 293 cells) after transfection, the viability of cells was checked by MTT assay and the

results are illustrated in figure 5. The cytotoxicity induced by HIV-1 Vpr or hIFN- γ is significant at 36 and 48 hr after transfection in HeLa or TE671 cells (48 and 72 hr in 293 cells) (Figure 5A, 5B, 5C). These results indicate that HIV-1 Vpr also has cytotoxicity in mammalian cell lines.

Induction of apoptosis by HIV-1 Vpr and hIFN- γ in human cell lines but not in chimpanzee, monkey or rodent cell lines.

Next, we wanted to investigate what kind of cytotoxicity could be induced by HIV-1 Vpr or hIFN- γ . There are two different cell death according to the morphological changes: necrosis and apoptosis. To determine if HIV-1 Vpr and hIFN- γ have apoptotic effects in different cell lines from various species, three human cell lines (HeLa, TE671, and 293), one chimpanzee cell line (PT1SK), one African green monkey cell line (Vero), and three rodent cell lines (CHTG49, A9, P3388D1) were used in this study. To avoid personal bias, these experiments were done by blind-coded. The transfection of different cell lines was done by Dr. L-J Chang and the processing of cells as well as the counting of apoptotic cells were done by Carlos Chen. Cells were transfected with tet-hGH-Vpr, tet-hGH-IFN- γ , tet-hGH-VSV-G, or an empty vector and VSV-G, or empty vector were used as negative controls and hIFN- γ was a positive control. The percentage of apoptotic cells were checked by Hoechst stain at 24, 36, and 48 hr after transfection (or 24, 48, and 72 hr in 293 cells). The results are illustrated in figure 6 (HeLa, TE671, and 293 cells) and summarized in table 4. It is demonstrated that only the human cell lines (HeLa, TE671 and 293 cells) but not the chimpanzee, monkey or rodent cell lines could be induced apoptosis by HIV-1 Vpr or hIFN- γ at 48 hr after transfection (Table 4).

In the case of human cell lines, it is shown that HIV-1 Vpr and hIFN- γ have similar kinetic effects in inducing apoptosis in HeLa and TE671 cells (Figure 6). In HeLa cells

(Figure 6A), the percentage of apoptotic cells is reached to 7.5% by HIV-1 Vpr, 6.5% by hIFN- γ and 2.5% by VSV-G protein at 48 hr after transfection. The result from VSV-G could be considered as background because VSV-G protein could not cause apoptotic cell death (95). In TE671 cells (Figure 6B), HIV-1 Vpr and hIFN- γ also have similar effects in inducing apoptosis as those seen in HeLa cells (compared with the empty vector). In the case of 293 cells, HIV-1 Vpr and hIFN- γ also induce apoptosis but in a slower kinetic manner than that in HeLa or TE671 cells.

The apoptotic effects induced by cycloheximide in different cell lines.

The results described above show that HIV-1 Vpr and hIFN- γ could only induce apoptosis in human cell lines but not in monkey, chimpanzee, or rodent cell lines. In order to know the sensitivity of apoptosis in each cell line and to get rid of the possibility that any cellular defects in inducing apoptosis existed in the cell lines we tested, a variety of cell lines described above were treated with 100 μ g/ml cycloheximide for 48 hr and the percentage of apoptosis induced by cycloheximide in each cell line was checked. The results are quantified and illustrated in figure 7. The percentage of apoptotic cells induced by cycloheximide in each cell line is 11.4% (HeLa), 16.8% (TE671), 10.2% (293), 11.9% (PT1SK), 15.2% (Vero), 14.5% (CHTG49), 27.1% (P388D1), 53.6% (H9) and 0.1% (A9) and the level of apoptosis in the cycloheximide non-treated cells in each cell line is not detected. H9 cell line was used as a positive control to show that everything was right in this system. Almost all of the cell lines from the different species were induced apoptosis by cycloheximide except for the A9 cells. In the case of A9 cells, we even increased the concentration of cycloheximide to 200 μ g/ml and the incubation time to 72 hr but still could not induce apoptosis in A9 cells. The results mentioned above show that most of the cell lines used for the Vpr apoptosis study could be induced apoptosis experimentally but not in the case of the mouse A9 cells.

hIFN- γ but not HIV-1 Vpr might have a paracrine/bystander effect in inducing apoptosis.

The results described above show HIV-1 Vpr and hIFN- γ have similar kinetic effects in inducing apoptosis in HeLa and TE671 cells. The hIFN- γ -induced apoptotic effects might be through both autocrine and paracrine mechanisms (results from Dr. Yuen Cui and Dr. L-J Chang, unpublished). In addition, it has been reported that the extracellular Vpr causes cell death by inducing the cell membrane permeability in yeast (96). So we wanted to investigate whether HIV-1 Vpr also have paracrine effect in inducing apoptosis. TE671 cells were transfected with 5 μ g of pHEF-1 α -hIFN- γ or pHEF-1 α -HIV-1 Vpr expression plasmids and empty vector as a negative control. The culture media were harvested at 48 hr after transfection from the transfected cells and then transferred to another fresh, non-transfected cells. The percentage of apoptosis in the non-transfected cells was analyzed at 24, 48, and 72 hr after culture supernatants were added. There is significant number of apoptosis induced by the supernatants of hIFN- γ -transfected cells but not by the supernatants of HIV-1 Vpr-transfected cells (compared with negative control). The results show that hIFN- γ but not HIV-1 Vpr induced apoptosis through a paracrine mechanism (Figure 8).

The experiment described above could only investigate the paracrine effects but not the bystander cell killing effects. To investigate if HIV-1 Vpr has bystander effects in inducing apoptosis at a single cell level, a reporter plasmid (pHEF-GFP) 2 μ g which encodes a green fluorescent protein was cotransfected with 5 μ g pHEF-HIV-1 Vpr, pHEF-hIFN- γ , or pHEF-HIV-1 Nef as a negative control into TE671 cells. 48 hr after transfection, cells were stained with Hoechst dye and counted for the percentage of apoptotic cells (blue fluorescence and condensed nuclei cells) as well as the percentage of GFP-positive (green fluorescence) cells under a fluorescence microscope by using two different filters as described in the Materials and Methods. The nuclei of the cells are blue after Hoechst

staining. However, only the transfected cells show green fluorescence because of the expression of GFP protein in the cells. The results are illustrated in figure 9 and show an example of single-colored (blue or green) cells or double-colored (blue and green) cells. A-C for negative control HIV-1 Nef (D-F for HIV-1 Vpr and G-I for hIFN- γ) are from the same view but by using two different color filters. A, D and G are under the Hoechst stain (blue color). B, E and H are fluorescent green produced by the GFP protein. C, F and I are double exposures and show whether the apoptotic cells are green (open arrow) or not (closed arrow). The percentage of apoptotic cells in transfected and non-transfected cell populations could show the paracrine and/or bystander killing effects. The percentages of apoptotic and transfected cells (green), and apoptotic and non-transfected cells (not green) in the total cell populations were calculated and the results are illustrated in figure 10. The percentages of apoptotic cells induced by hIFN- γ or HIV-1 Vpr in the total cell populations are about 17.4% and 19.1%, respectively. In addition, the percentages of apoptotic/transfected (green) cells and apoptotic/non-transfected (not green) cells induced by hIFN- γ or HIV-1 Vpr in the total cell populations are 11.8% and 5.6%, 16.5% and 2.6%, respectively. On the other hand, the percentages of apoptotic/transfected (green) cells induced by hIFN- γ or HIV-1 Vpr in the transfected (green) cells populations are about 22.1% or 30.8%, respectively. The majority of apoptotic cells induced by HIV-1 Vpr are transfected (green) cells (approximately 87%). In contrast, a significant number of apoptotic cells induced by hIFN- γ are non-transfected cells (approximately 35%). Conclusively, the results described above indicate that hIFN- γ induced apoptosis through both autocrine and paracrine/bystander routes but it was not so apparent for HIV-1 Vpr.

Finally, we wanted to examine whether the cell-cell interaction was required by the hIFN- γ -induced paracrine and/or bystander apoptotic effects by using a ^3H -thymidine release assay. In order to know the levels of apoptotic cells could be reflected by this method, cells were treated with cycloheximide as positive control. Cells were split into a

24-well plate, and labeled with ^3H -thymidine for 16 hr. Different concentrations of cycloheximide were added into cultured cells and the amount of ^3H -thymidine released into the supernatant was measured at 24, 48 hr after cycloheximide treatment. The percentages of apoptosis were calculated by the ratios of the amount of ^3H -thymidine in the supernatant subtracted the amount of ^3H -thymidine spontaneously released to the total amount of ^3H -thymidine absorbed by the cells. The percentages of cycloheximide-induced apoptosis determined by the ^3H -thymidine release assay or Hoechst stain are compared and illustrated in Table 5A. The percentages of apoptosis determined by ^3H -thymidine release assay is apparently lower than those by the Hoechst stain.

Next, TE671 cells were transfected with 5 μg pHEF-HIV-1 Vpr, pHEF-hIFN- γ or negative control pHEF-HIV-1 Nef expression plasmid. At 24 hr after transfection, cells were harvested and cocultured with the non-transfected cells labeled with ^3H -thymidine for 16 hr previously in a 24-well plate. The percentages of apoptosis in the non-transfected cells were examined at 24 hr, 36hr, 48 hr, 60 hr and 72 hr after coculturing. According to the amount of ^3H -thymidine released into the supernatants, there was no apparent apoptosis induced by the HIV-1 Vpr-transfected, or the hIFN- γ -transfected cells in the ^3H -thymidine-labeled cell populations (Table 5B).

The mechanisms of HIV-1 Vpr in inducing apoptosis might be related to those of the Bcl-2 family members.

A variety of cellular factors have been shown to be involved in the signal pathways of apoptosis. For instance, the Bcl-2 family members have been well documented to participate in the signaling pathway of apoptosis (97). Some of the members block apoptosis (e.g. Bcl-2 and Bcl-X_L) but some other members enhance the apoptosis process (e.g. Bax and Bcl-X_s). In order to further investigate the possible mechanisms of HIV-1

Vpr in inducing apoptosis and the potential cellular factors assessed for their influences on the apoptotic effects of HIV-1 Vpr, pHEF-HIV-1 Vpr expression plasmid was cotransfected with different constructs of Bcl-2 family members (e.g. Bcl-2, Bax, Bcl-X_L and Bcl-X_s) which were cloned into an eukaryotic expression vector. To avoid personal bias, this experiment was done blind-coded. Different transfection combinations were performed by Dr. L-J Chang, the processing of cells and the percentages of the apoptotic cells were performed by Carlos Chen. The results are shown in figure 11. When compared with the percentages of apoptosis in the HIV-1 vpr-transfected cells (cotransfected with a control vector cDNA3), the percentages of apoptosis in the cells cotransfected with HIV-1 vpr as well as Bcl-2/Bcl-X_L are reduced significantly ($P < 0.05$, t test). However, the apoptotic effects of HIV-1 Vpr are recovered significantly ($P < 0.05$) when cells are cotransfected with HIV-1 Vpr, Bcl-2, Bax / or HIV-1 Vpr, Bcl-X_L, Bcl-X_s respectively. It is suggested that Bax or Bcl-X_s could neutralize the suppression of Bcl-2 or Bcl-X_L on the apoptotic effects of HIV-1 Vpr. On the other hand, Bax or Bcl-X_s could not further enhance the apoptotic effects induced by HIV-1 Vpr significantly ($P > 0.05$, t test). The results described above indicate that the possible mechanisms of HIV-1 Vpr in inducing apoptosis might be related to the Bcl-2 family members such as Bcl-2/Bcl-X_L or Bax/Bcl-X_s.

The absence of apoptotic effects in the expression of HIV-2 and SIVmac239 Vpr and/or Vpx, or SIVagm Vpx in mammalian cell lines from different species.

In order to examine whether the apoptotic effects found in HIV-1 Vpr could be extended to the Vpr or Vpx of other lentiviruses such as HIV-2ROD, SIVmac239 or SIVagm, the vpr and vpx genes of HIV-2ROD, SIVmac239 or SIVagm were cloned into an eukaryotic cell expression vector pHEF1 α . The apoptotic effects of each construct were investigated

by transient transfection in different cell lines as described before. The percentages of apoptosis were checked at 48 hr after transfection and the results are shown in table 6. When these constructs were transfected into each of the cell lines individually, there were no apoptotic effects found in any of these cell lines. Next, we cotransfected vpr and vpx of HIV-2_{ROD} or SIV_{mac239} expression plasmids into different cells since both of the vpr and vpx genes exist in the genomes of HIV-2_{ROD} and SIV_{mac239}. Again, there was no apparent apoptosis could be detected in any of the cell lines we tested. The transfections of these constructs were repeated three times in PT1SK, Vero, CHTG49, P388D1, and A9 cells and six times in HeLa, TE671, and 293 cells. The transfection efficiency was checked routinely by measuring the amount of human growth hormone in the media. The results described above suggest that the vpr or vpx genes from other lentiviruses could not induce apoptosis especially those from the non-human primate lentiviruses in the chimpanzee or African green monkey cell lines (Table 6).

Chapter IV.

Conclusion and Discussion

The establishment of a tetracycline inducible system.

A variety of inducible gene expression systems have been established in order to meet the requirements for the regulation of gene expression. Most of the methods for inducing gene expression such as metallothionein, heat shock, or steroid-control always meet with problems either pleiotropic interference effects on the host genes or high basal levels of gene expression (98). Recently, there are two systems appears to have overcome these problems: the tetracycline (99,100) and the progesterone antagonist (RU486) (101) inducible systems. These systems control the expression of genes by using transactivation factors and DNA response elements from bacteria. As the results have suggested, the regulation of gene expression is specific, the host genes are not influenced, and there is no toxicity to cells. In addition, the expression of genes can be modulated by a very broad range of concentrations of extracellular factors such as tetracycline or the progesterone antagonist (RU486).

In the study of HIV-1 Vpr, we originally attempted to establish stable transfection cell lines by using a tetracycline-inducible gene expression system in different cell lines such as HeLa and TE671 cells (by Dr.L-J Chang and Jergen Chu). After the HIV-1 vpr expression plasmids are transfected into cells, the expression of vpr gene has to be blocked by using tetracycline in order to prevent the interference with cell growth due to the cytostatic effect of the Vpr protein. In our hand, the establishment of stable transfection cell lines was unsuccessful because the basal levels of gene expression were high and the induction levels were not very high (proximately about 30 fold, from Dr.L-J Chang, Jergen Chu). In

contrast, the results published before (102,103) showed that the range of induction varied from 100 to 1000 fold in HeLa cells at the same concentration of tetracycline that we used. Once the expression of the vpr gene starts even in the presence of tetracycline, cell growth will be interfered with and it would not be easy to get stably-transfected cell lines. Thus, we changed to another strategy to study Vpr protein by using a transient transfection system. In this transient transfection system, we met the same problems as those in the stable transfection system before. The basal level of gene expression was too high and the induction level was even worse than that in the stable transfection system. So all transfections with pNREP-hGH-derived constructs in our transient transfection system were done without tetracycline in culture medium. There are several reasons for the high basal level of gene expression. First, different cellular backgrounds of different cell lines may have different cellular factors which could by-pass the control of the system and activate gene expression. Second, a part of the intracellular plasmids may integrate into the chromosome of the transfected cells. If the target gene is inserted into the position near an endogenous enhancer element, this endogenous enhancer could activate the expression of the target gene even in the presence of tetracycline and results in a very high basal level of gene expression as well as the low induction fold.

The assay of cell viability : MTT/Trypan blue exclusion method.

MTT and trypan blue exclusion method are two popular methods in the study of cell viability. In the trypan blue exclusion method, trypan blue is able to enter into dead cells and the dead cells become blue-colored because the membrane permeability is lost in dead cells. So the viability of cells are calculated by counting the percentage of blue color-exclusive (alive) cells in the total cell population under microscope. In the MTT assay, the viability of cells is determined by the rate of the cellular bioreduction of MTT with enzymes of the endoplasmic reticulum and mitochondria. The purple-colored particles are metabolites from

MTT and their amounts in the medium are measured by the optical density (O.D.) in an ELISA reader. If the viability of non-treated cells is considered as 100%, the viability of cells treated under different situation is calculated by the percentage of O.D.(treated cells) to O.D.(non-treated cells). We found that the trypan blue exclusion method could not be used properly especially in a short period of time after transfection. It is possible that the membrane permeability increased by transfection solution could not be recovered in a short time period after transfection, so the trypan blue dye could enter into the cells which are still alive. Since trypan blue exclusion method could not be used properly especially at 24 hr after transfection in our transient transfection system, we chose the MTT method as our standard cell viability assay method.

The apoptosis analysis by Hoechst, Propidium iodide, and Tunel staining.

Hoechst, Propidium iodide, and Tunel stain (92-94) are popular methods in the study of apoptosis. With the Hoechst stain, Hoechst dye specifically binds to the chromatin and shows the nuclei of cells to be blue when checked under a fluorescence microscope. When cells are induced to become apoptotic, the morphology of the nucleus changes from an intact-round shape to a few condensed-round spots (92).

In the Propidium iodide stain, cells usually need to be permeabilized in 70% ethanol before being stained to improve the cell membrane permeability because of the size of the dye particles. After the PI staining, the percentages of apoptotic cells are determined by flow cytometry. Once cells become apoptotic, the histograms of forward scatter in flow cytometry would be decreased corresponding to the reduction in sizes of the apoptotic cells and those of DNA fluorescence would be reduced due to the increased difficulty of the Propidium iodide dye into the condensed-nucleus (93).

In the Tunel stain, a biotin-dUTP can be specifically labeled to the 3'-OH of the broken ends in a chromosome by terminal deoxynucleotidyl transferase (TdT). Then, an avidin-neutralite fluorescence can be conjugated to it. DNA fragmentation is a very typical morphological change of the nucleus when cells become apoptotic. If there are more apoptotic cells, there would be more DNA broken ends produced, and more fluorescence-shifting would be detected (94).

Originally, we tried to compare the results of different apoptosis staining methods to see if they correlated with each other. After the induction of apoptosis by cycloheximide treatment, the results from the three different methods did correlate to each other. In contrast, the results of these methods in the transient transfection system were not consistent. The results of PI stain and Tunel methods were considered extremely unreliable when compared with those of Hoechst stain because very high backgrounds of apoptotic cells were shown even in the negative controls. The possible reason for this may be the interference of intracellular plasmid DNA in the transfection. The PI dye and dUTP may not be able to distinguish the chromosome or the intracellular plasmid DNA. In contrast, the apoptotic cells could be distinguished very easily by the typical morphological change under a fluorescence microscope. The nuclei of normal cells would be round-shaped when checked under a fluorescence microscope after Hoechst dye staining. In contrast, the nuclei of apoptotic cells could not keep intact and a few bright spots would exist in the nuclei of cells. Only cells with three or more chromatin fragments (bright spots) were considered as apoptotic cells. We could not find the reason and overcome the problems of apoptosis assay from PI and Tunel stain in our transient transfection system finally. So we decided to use Hoechst stain as our standard apoptosis assay method.

The differential apoptosis effects among Vpr, Vpx in the mammalian cultured cells.

The results described above show that only HIV-1 Vpr could induce apoptosis in human cell lines such as HeLa, TE671, and 293 but not in chimpanzee cells (PT1SK), African green monkey cells (Vero), and rodent cells (CHTG49, A9, and P388D1). In addition, the Vpr and/or Vpx from simian immunodeficiency viruses would not cause any apoptosis either in human cells or in chimpanzee/monkey cells. Since all of the cell lines from different species, except A9 cells, could be induced apoptosis by cycloheximide experimentally, the potential cellular defects in inducing apoptosis might not be the reason why the cell lines from chimpanzee, monkey, or rodent we tested could not be induced apoptosis by HIV-1 Vpr or hIFN- γ .

Different cellular backgrounds may be the reason for different levels of apoptosis induced by HIV-1 Vpr in different human cell lines.

HIV-1 Vpr induces apoptosis with similar kinetics in HeLa, TE671 and 293 cells. However, the levels of apoptosis induced by HIV-1 Vpr at any given time points in these three cell lines are different. It seems that there are some common mechanisms for HIV-1 Vpr to cause apoptosis in these cell lines but the different levels of apoptosis induced by HIV-1 Vpr may be due to the different cellular backgrounds of these cells. Bcl-2 and NF- κ B may be two candidates to explain this because they both have been well documented to be involved in the signaling pathways of apoptosis (104,105).

Bcl-2 is a proto-oncogene found in chromosome segment 18q21. The gene product is localized in the outer mitochondria membrane and has been shown to protect cells against a variety of apoptotic stimuli such as glucocorticoids, γ -irradiation, phorbol esters,

ionomycin, and the withdrawal of some growth factors (104). On the other hand, the transactivation factor NF- κ B (or nuclear factor κ B) has been shown to be involved in apoptosis but the results are still controversial (105). It has been reported that NF- κ B can enhance apoptosis under different apoptosis stimuli. It has been shown that the induction of NF- κ B activity is needed to induce apoptosis in the serum-starved human embryonic kidney cells (106), in the prostate-cancer cells under a oxidative stress caused by anti-cancer drugs, in the irradiated-fibroblast cells taken from the ataxia telangiectasia (AT) patients (107), and in the developing chicken embryos and bone-marrow cells (108). In contrast, it has been reported recently that NF- κ B could prevent the apoptosis induced by TNF- α although the induction of NF- κ B activity can be enhanced by TNF- α (109). It is suggested that some chemotherapy agents and TNF- α actually could induce two different signaling pathways: one is to induce apoptosis and the other is to induce the activity of NF- κ B, and then the latter would block the function of the former in inducing apoptosis (110). So, different levels of some cellular factors such as Bcl-2 and NF- κ B in these cells may influence on the levels of apoptosis induced by HIV-1 Vpr or hIFN- γ in HeLa, TE671, and 293 cells.

Does the action of HIV-1 Vpr on the cell cycle arrest lead to the Vpr-mediated apoptosis ?

Is it possible that the induction of apoptosis by HIV-1 Vpr is an indirect effect of Vpr-mediated cell cycle arrest ? It has been reported that the Vpr protein of HIV-1, HIV-2/SIVmac239/SIVsm induces cell cycle arrest at the G2/M phase in human HeLa cells and in African green monkey CV-1 cells (related to the Vero cells we used) and both Vpr of SIVsyk and Vpx of SIVagm can induce cell cycle arrest in CV-1 cells but not in HeLa cells (13,111). On the other hand, the results of our apoptotic study show that HIV-1 Vpr can induce apoptosis only in HeLa cells but not in Vero cells even though it causes cell cycle arrest in

both cell lines (13). Thus, it appears that the induction of apoptosis by HIV-1 Vpr may not derive from the cell cycle arrest and the mechanisms of cytostatic and cytotoxic effects of HIV-1 Vpr may be distinct from each other.

The paper published recently by Dr. Irvin Chen's group also showed that HIV-1 Vpr induces apoptosis in human fibroblasts, T cells, and HeLa cells (112). It has been shown that the induction of apoptosis can not be abrogated even though the cell cycle was restarted by using agents to release the cell cycle arrest caused by Vpr. It is likely that the maintenance of cell cycle arrest at the G2/M phase was not necessary for the HIV-1 Vpr induced-apoptosis. In addition, the ratios of hyperphosphorylated form of p34^{CDC2} kinase related to the HIV-1 Vpr-induced cell cycle arrest stayed constant and they did not correlate with the kinetics of apoptosis induced by Vpr. The results described above also support our hypothesis that HIV-1 Vpr-induced apoptosis may occur independently from the cell cycle arrest and the mechanisms for both may be distinct.

The induction of apoptosis by hIFN- γ and HIV-1 Vpr may be through differential routes.

The results from our study show that hIFN- γ induces apoptosis via both autocrine and paracrine/by-stander routes (by Dr. Yuen Chu and Dr. L-J Chang). In contrast, HIV-1 Vpr may not induce apoptosis via paracrine/by-stander routes. However, it has been reported that extracellular HIV-1 Vpr could increase the cell membrane permeability and eventually cause cell death in yeast cell (96). Why is it that the same effects was not seen when the supernatant from the HIV-1 Vpr transfected cells was transferred into the non-transfected cells? It is possible that the amount of HIV-1 Vpr released into the supernatant is low because Vpr is an intracellular nuclear protein.

In the ^3H -thymidine release experiment, after coculturing hIFN- γ -transfected or HIV-1 Vpr-transfected cells with the non-transfected cells, no induction of apoptosis was observed in the non-transfected cells at any given time points. It is possible that this is caused by the limitation of the method. It was demonstrated that the results from the Hoechst stain were always higher than those from the ^3H -thymidine release assay. Apoptotic cells, whether in the early stage or in the late stage of apoptosis, can be determined by Hoechst staining under a fluorescence microscope according to the morphological changes. However, it is impossible to detect the apoptotic cells by the amount of the ^3H -thymidine released into the supernatants unless the apoptotic cells are in the late stage and broken. So low levels of apoptosis may not be detected by ^3H -thymidine release assay and that might be the reason why the induction of apoptosis did not be detected in the non-transfected cells when cocultured with the hIFN- γ -transfected cells. Nevertheless, we still can not ignore the other possibility that the induction of apoptosis by hIFN- γ does not require the cell-cell interaction.

The influences of Bcl-2 family members on the apoptotic effects of HIV-1 Vpr.

The analysis of possible roles of Bcl-2 family members in HIV-1 Vpr-induced apoptosis suggests that apoptosis induced by HIV-1 Vpr might go through the Bcl-2-related signaling pathways. There are at least 5 members in the Bcl-2 family, which include Bcl-2, Bcl-X_L, Bcl-X_s, Bax, and BAD (104). Some of the members inhibit apoptosis e.g. Bcl-2, Bcl-X_L. In contrast, some can enhance apoptosis e.g. Bcl-X_s and Bax, and some members act as regulators e.g. BAD.

Most of the studies suggest that the Bcl-2 family members have to be in the mitochondria to regulate apoptosis (50). Bcl-2 and Bcl-X_L could regulate the cellular

localization of apoptotic factors in the cells. For instance, Bcl-X_L could retain the death-regulatory factor ced-4 in the cytosol and prevent cells from undergoing apoptosis (58). When the apoptotic signal cascades start, cytochrome c is released from the mitochondria to the cytosol (113) which activates the activity of an ICE-like protease, Yama/CPP32 (114). It has been reported that Bcl-2 could inhibit the release of cytochrome c from the mitochondria to the cytosol and further inhibit the function of Yama/CPP32 (113). Since the results of our studies show that the Bcl-2 family might be involved in the HIV-1 Vpr-mediated apoptosis, it is possible that HIV-1 Vpr induces apoptosis by stimulating the translocation of cytochrome c from the mitochondria to the cytosol and further activating the function of Yama/CPP32 in the HIV-1 Vpr-transfected cells.

Other possible mechanisms related to the induction of apoptosis by HIV-1 Vpr.

During the cell cycle arrest, it has been shown that Vpr could inhibit the function of p34^{CDC2} by binding and disrupting the function of the upstream factor, PP2A, but the results from different laboratories are controversial (81). In addition, by using Vpr as a conjugated ligand in an affinity column, some cellular factors have been identified to bind to Vpr such as the glucocorticoid receptor (GR) (115), the 41-kDa Vpr-interacting protein 1 (Rip-1) (115), and the uracil DNA glycosylase (UNG) (116,117).

In the case of the glucocorticoid receptor, it has been shown that Vpr could bind glucocorticoid receptor through an unknown 41-kDa cellular protein Rip-1 and the physical interaction between them could be blocked by the glucocorticoid antagonist mifepriston (RU486) (115). In addition, it is known that the glucocorticoids such as dexamethasone are able to induce apoptosis in mature and activated T cells (118). It is suggested that the glucocorticoid-induced apoptosis is caused by interfering with the expression of other

transcriptional factors required cell survival through the binding between glucocorticoid and its receptor (119). It is possible that HIV-1 Vpr induces apoptosis by binding the glucocorticoid receptors distributed in the cytosol, translocating from the cytosol to the nucleus, and acting as a transactivation factor to inhibit or enhance the transcription of specific genes related to the control of cell survival.

In the case of uracil DNA glycosylase (UNG), it has been shown that UNG could interact with the Vpr protein of HIV-1 and SIVsm (116,117). However, a set of Vpr mutants showed that the binding abilities of Vpr mutants with UNG did not correlate to the capabilities of Vpr mutants in inducing the cell cycle arrest. Because the UNG protein is a DNA repair enzyme, it is possible that HIV-1 Vpr causes apoptosis by binding UNG, and interfering with the functions of UNG in DNA repair.

Vpr has been shown to form a cation-selective channel in planer lipid bilayers in an artificial chamber system (120). It is also possible that Vpr might form an ion channel in the intracellular membranes such as nuclear, mitochondria, endoplasmic reticulum *in vivo*, and that might be important for the induction of apoptosis.

The pathogenic and non-pathogenic infection of human and non-human models by HIV/SIV.

The infection of CD4-positive cells by HIV-1 often causes acute cytopathic effects such as syncytia formation (32), single cell lysis (33) and some chronically cytopathic effects such as apoptosis (121,122). The acute cytopathic effects of HIV-1 can not explain the discordance of pathogenic and non-pathogenic infection of HIV-1 in human and non-human primate models (123,124). The acute cytopathic effects also exist in the non-pathogenic primate models such as HIV-1-infected chimpanzees and naturally SIV-infected African green

monkeys. In contrast, a high level of apoptosis could only be found in the pathogenic primate models such as SIV-infected macaques and HIV-1-infected humans who develop AIDS but not in HIV-1-infected chimpanzees which could not develop AIDS (42). Such correlation suggests that the induction of apoptosis might be the main mechanism for the depletion of CD4-positive cells in AIDS patients (43).

As compared with the results from pathogenic and non-pathogenic primate models described above and our results, it makes sense that HIV-1 Vpr could not induce apoptosis in the PT1SK chimpanzee cells because the level of apoptosis found in HIV-1-infected chimpanzees is very low (42,43). Further experiments could be done to see whether vpr and or vpx gene of SIV could induce apoptosis in the cell lines from some Asian macaque species such as rhesus, cynomolgus, stumptailed and pigtailed macaques because high level of apoptosis could be found in Asian macaques and it is related to the disease progression.

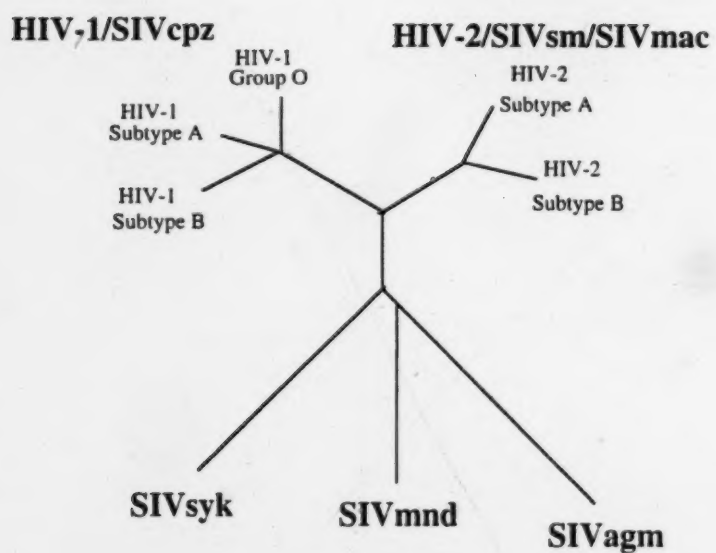


Figure 1. The phylogenetic relationships of Vpr protein sequences.

Information contained in this figure was obtained from reference 111:

M. Emerman et al. 1997 and has been modified.

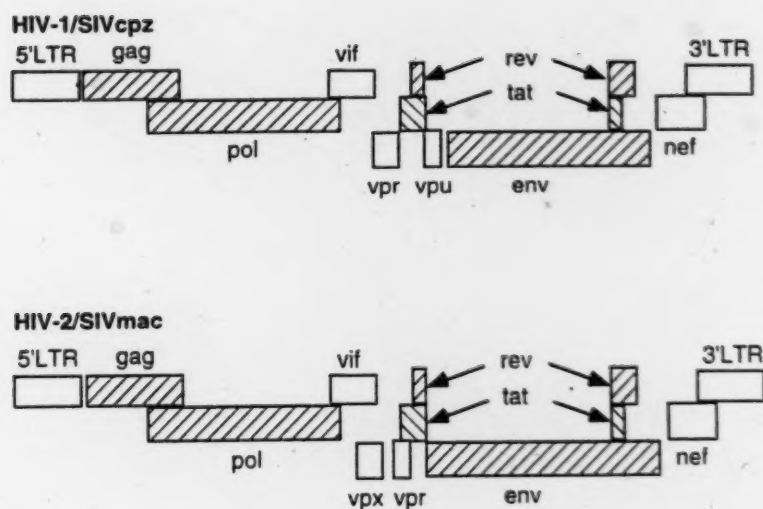
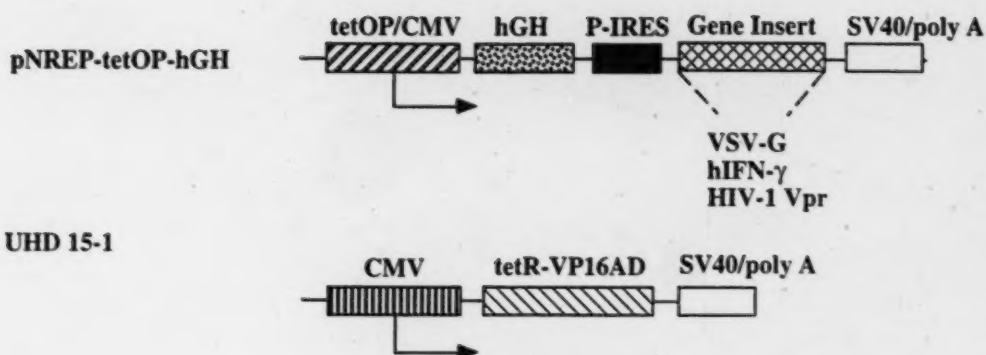


Figure 2. The genome organization of primate lentiviruses.
Information contained in this figure was obtained from reference 125
: B.N. Fields et al., 1996 and has been modified.

A.



B.

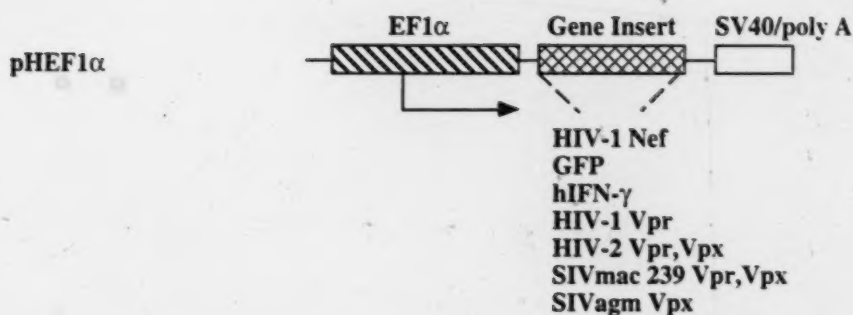


Figure 3. Diagram of the heterologous expression constructs:

A. The vectors of the tetracycline inducible system. The pNREP-tetOP-hGH expression vector controlled by the binding of the tTA protein to tetOP site was used for the cloning of HIV-1 Vpr, hIFN- γ , or VSV-G. The UHD 15-1 vector controlled by a CMV promoter expresses a fusion protein tetR-Vp16AD (tTA protein). P-IRES, internal ribosome entry sequences derived from poliovirus. Poly A, polyadenylation signal of SV40.

B. The pHEF1 α expression vector controlled by the EF1 α promoter which is from the human elongation factor 1 α .



Figure 4: The morphological difference between normal and apoptotic cells. 100 $\mu\text{g/ml}$ of cycloheximide was used to induce apoptosis in HeLa cells. 48 hr after treatment, cells were harvested, fixed, and stained with Hoechst dye and checked under a fluorescence microscope as described in the Materials and Methods. The morphology of nucleus in normal cells is an intact-round shape (closed arrow). However, the morphology of nucleus in apoptotic cells becomes a few condensed-round spots (open arrow). Only cells with three or more chromatin fragments (bright spots) are considered apoptotic.

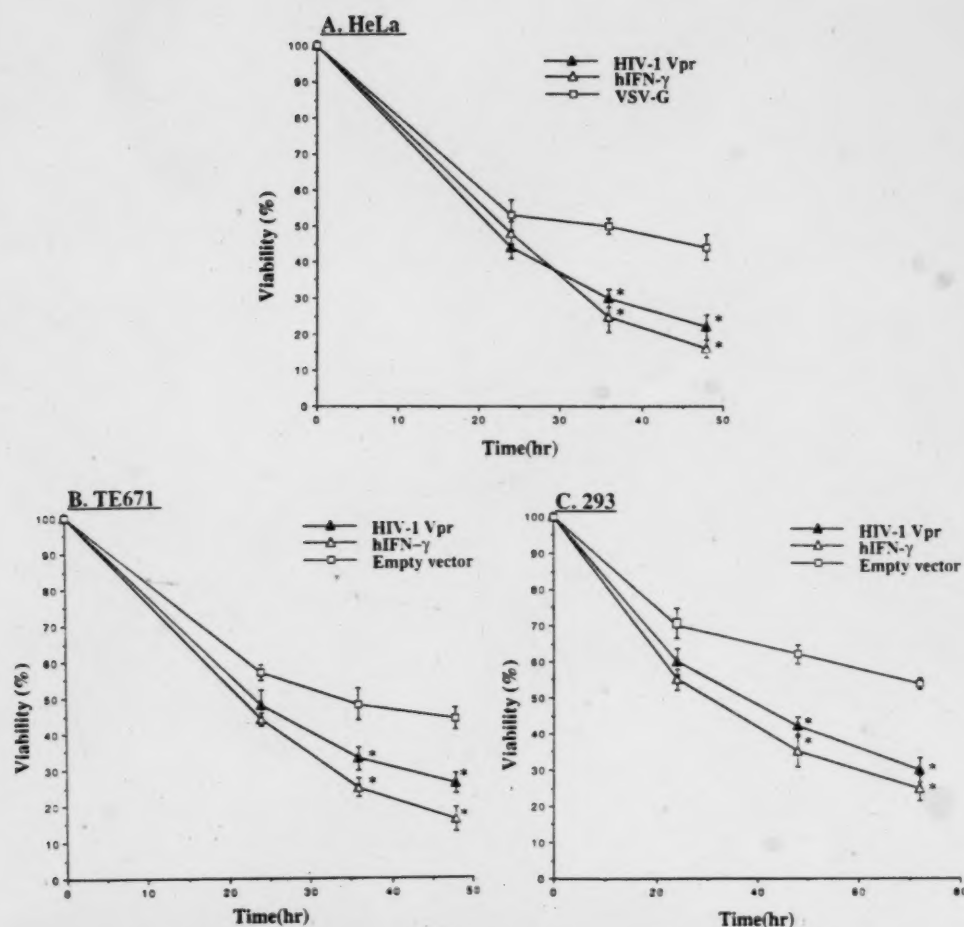


Figure 5 : The kinetics of cytotoxicity induced by HIV-1 Vpr or hIFN- γ in (A) HeLa cells (B) TE671 cells and (C) 293 cells.

Cells were transfected with 5 μ g of tet-hGH-derived expression plasmids and 2 μ g UHD 15-1, harvested at 24, 36, and 48 hr (24, 48, 72 hr in 293 cell) and the viability of cells was determined by MTT assay as described in the Materials and Methods. Each bar represents the mean $\pm$ SD and '*' means the percentage of cell cytotoxicity is significantly different from that induced by the negative control e.g. VSV-G or empty vector ($p < 0.05$ t test, A; $n=5$, B; $n=4$, C; $n=3$).

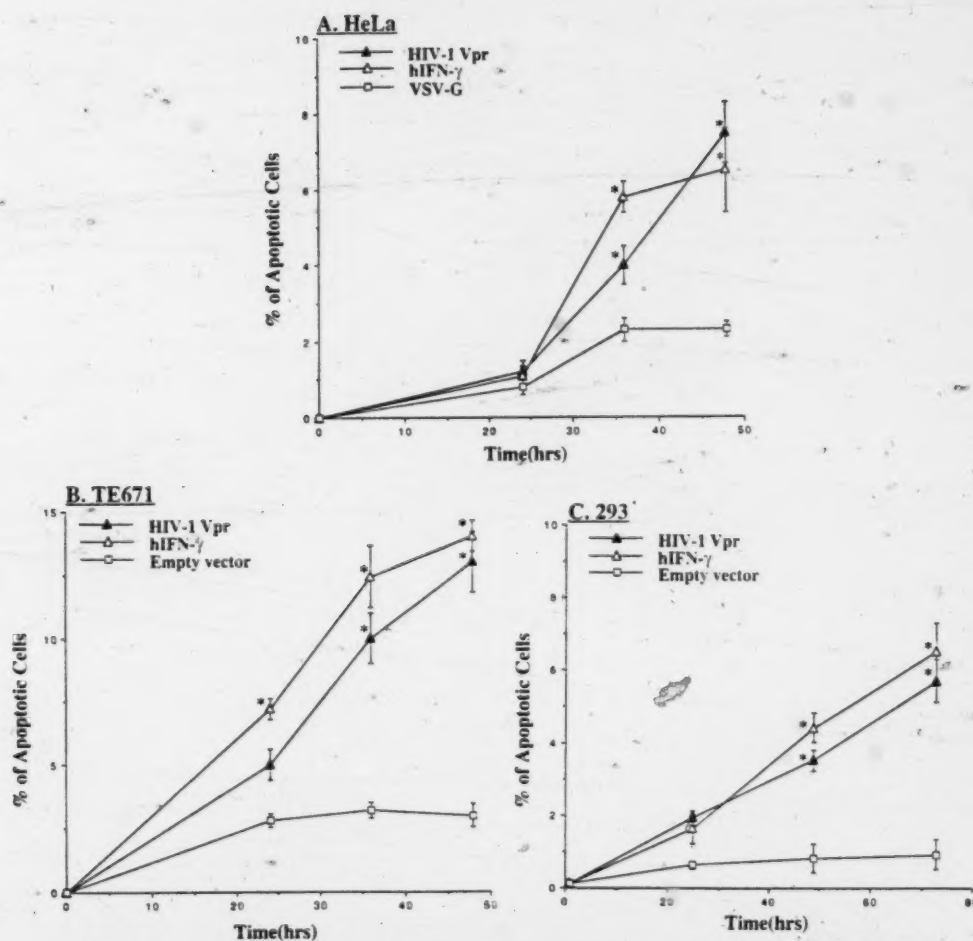


Figure 6 : The kinetics of apoptosis induced by HIV-1 Vpr or hIFN- γ in (A) HeLa cells (B) TE671 cells and (C) 293 cells.

Cells were transfected with 5 μ g of tet-hGH-derived expression plasmids and 2 μ g UHD 15-1, harvested at 24, 36, and 48 hr (24, 48, 72 hr in 293 cell), fixed and stained with Hoechst dye, and counted for the number of apoptotic cells under a fluorescence microscope as described in the Materials and Methods. Each bar represents the mean $\pm$ SD and '*' means the percentage of apoptosis is significantly different from that induced by negative control, e.g. VSV-G or empty vector ($p < 0.05$ t test, A; $n = 5$, B; $n = 4$, C; $n = 3$).

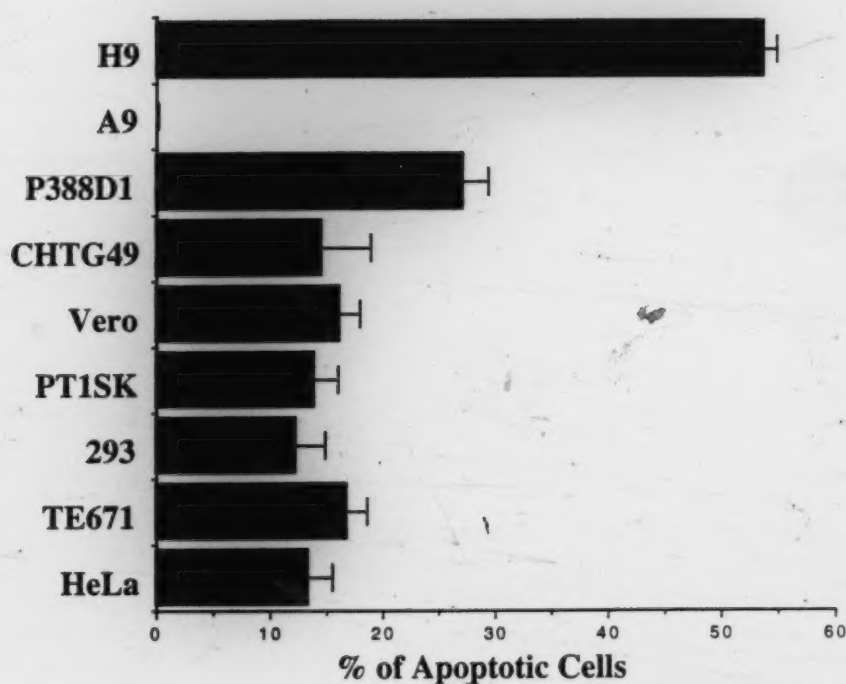


Figure 7: The apoptotic effects of cycloheximide in different cell lines. 100 μ g/ml of cycloheximide was used to induce apoptosis in different cell lines. 48 hr after treatment, cells were harvested, fixed, and stained with Hoechst dye and counted for the percentages of apoptotic cells under a fluorescence microscope as described in the Materials and Methods. Each bar represents the mean $\pm$ SD (n=4).

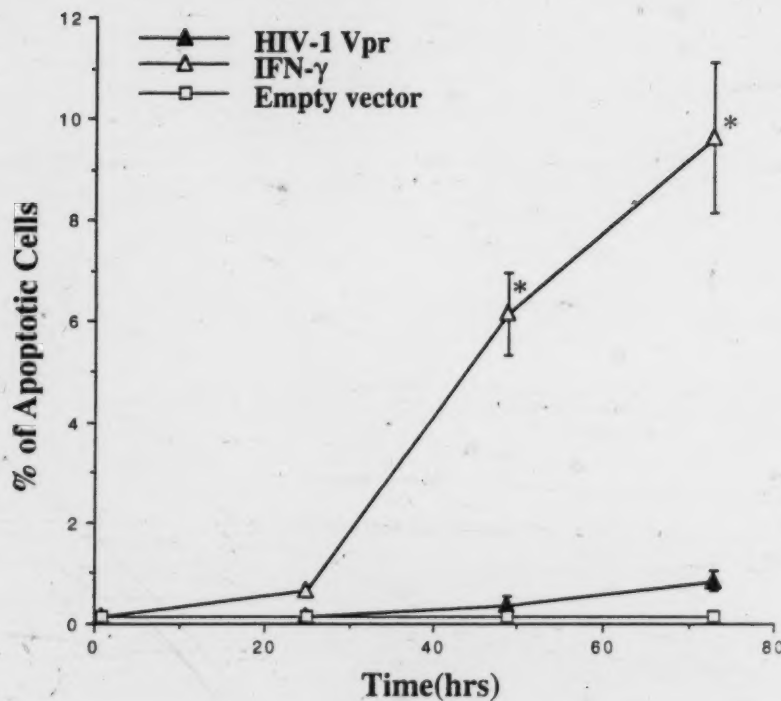
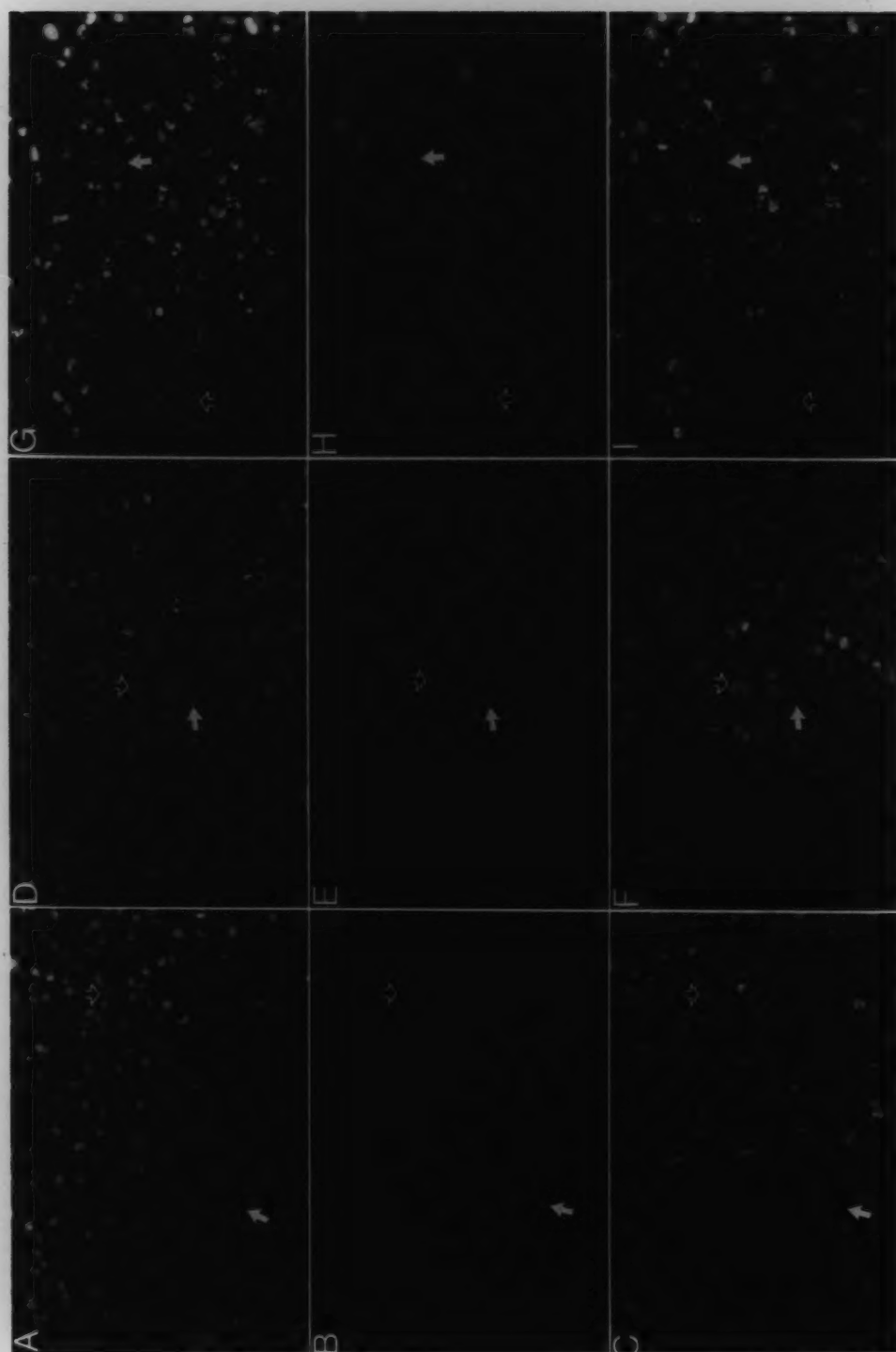


Figure 8 : The kinetics of apoptosis induced by extracellular hIFN- γ or HIV-1 Vpr in TE671 cells.

Cells were transfected with 5 μ g of pHEF1 α -derived expression plasmids. 48 hr after transfection, the culture supernatants were harvested from the transfected cells and transferred to other fresh non-transfected cells. Cells were harvested at 24, 48 and 72 hr, fixed, stained with Hoechst dye and the percentages of apoptotic cells was counted under a fluorescence microscope. Each bar represents the mean $\pm$ SD and '*' means the percentage of apoptosis is significantly different from that induced by the negative control e.g. empty vector ($p < 0.05$, t test, $n = 3$).

Figure 9: Hoechst dye staining and GFP expression of transfected TE671 cells.

The pHEF-derived HIV-1 Vpr, hIFN- γ or HIV-1 Nef (negative control) plasmid 5 μ g were cotransfected with 2 μ g of pHEF-derived GFP expression plasmid in each sample. 48 hr after transfection, cells were harvested and counted for the percentages of apoptotic/GFP+(green) cells (open arrow) in the total/or transfected (green) cell population and apoptotic/GFP-(not green) cells (closed arrow) in the total cell populations respectively. A to C, HIV-1 Nef (negative control), D to F, hIFN- γ , and G to I, HIV-1 Vpr. A, D and G: Hoechst stain (blue cells); B, E and H: GFP+cells (green cells); C, F and I: double colored cells under two different color filters.



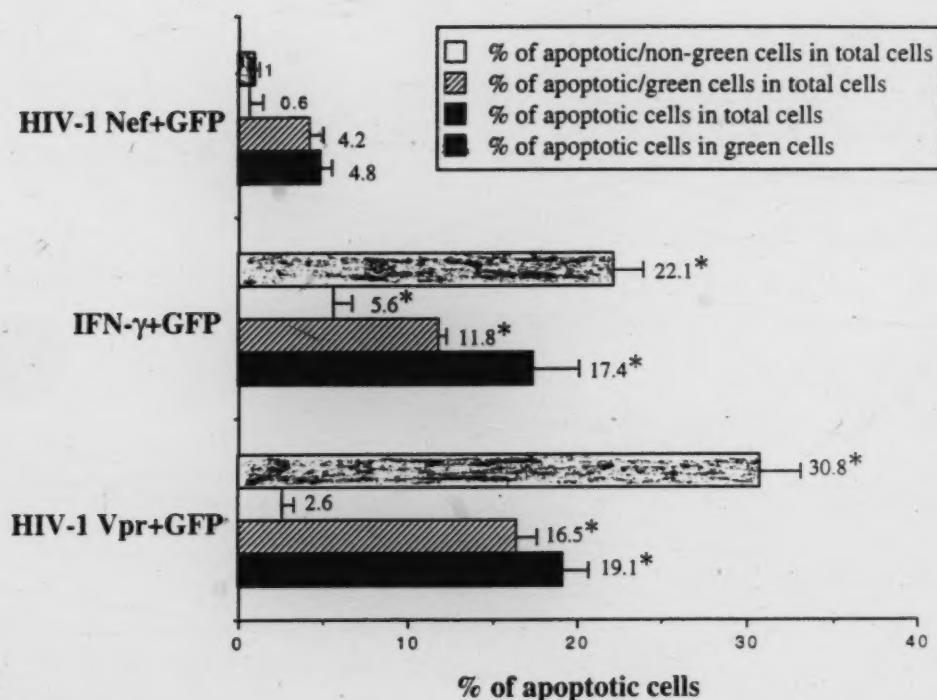


Figure 10: The quantitative analysis of paracrine/bystander apoptotic effects induced by HIV-1 Vpr and IFN-γ in TE671 cells.

The pHEF-derived HIV-1 Vpr, hIFN-γ or HIV-1 Nef (negative control) plasmids 5 μg were cotransfected with 2 μg of pHEF-derived GFP expression plasmid in each sample. 48 hr after transfection, cells were harvested and counted for the percentages of apoptotic cells, the percentages of apoptotic/GFP+(green) or apoptotic/GFP-(not green) cells in the total cell populations, and the percentages of apoptotic cells in the green cell populations (black, striped, white, and grey bar, respectively). Each bar represents the mean ± SD and '*' means the result is significantly different from that induced by the negative control e.g. HIV-1 Nef in each group ($p < 0.05$ t test, $n=4$).

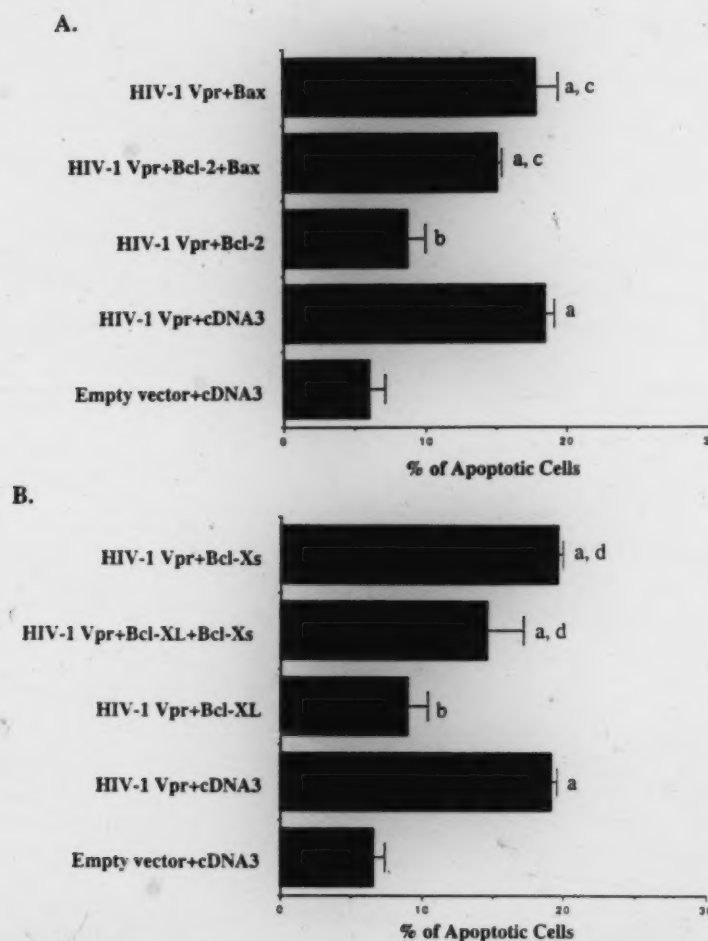


Figure 11: The effects of Bcl-2 family proteins on the HIV-1 Vpr-induced apoptosis in TE671 cells.

The pHEF-derived HIV-1 Vpr expression plasmid 5 μ g was cotransfected with (A) either 3 μ g of Bcl-2 or Bax expression plasmid or both. (B) either 3 μ g of Bcl-XL or Bcl-Xs expression plasmid or both. Cells were harvested at 48 hr after transfection and counted for the percentages of apoptotic cells by Hoechst stain as described in the Materials and Methods.

'a' means the results are significantly different from that of empty vector+cDNA3; 'b' means the results are significantly different from that of HIV-1 Vpr +cDNA3; 'c' means the results are significantly different from that of HIV-1 Vpr + Bcl-2; 'd' means the results are significantly different from that of HIV-1 Vpr + Bcl-XL ($p < 0.05$, t-test, $n = 4$). Each bar represents the mean $\pm$ SD ($n = 4$).

Table 1. The oligodeoxyribonucleotide primers used for PCR amplification.

HIV-1 NL4-3 Vpr
5' primer : 5'-CAGAGGACATATGGAACAAGCC-3'
3' primer : 5'-CCCGGATCCTAGGATCTACTGGCTCC-3'
HIV-1 NL4-3 Nef
5' primer : 5'-CCCGGATCCGTATGGGTGGCAAGTGGTC-3'
3' primer : 5'-ACCTGCAGTCAGCAGTTCTTGAAGTAC-3'
HIV-2 ROD Vpr
5' primer : 5'-CTCTCTCATATGGCTGAAGCACCAACA-3'
3' primer : 5'-AAACGGATCCTTATTGCATGTTTCTAGG-3'
HIV-2 ROD Vpx
5' primer : 5'-CTCTCTCATATGACAGACCCCAGAGAG-3'
3' primer : 5'-AAACGGATCCTTAGACCAGACCTGGAGG-3'
SIVmac239 Vpr
5' primer : 5'-CAGAGGACATATGGAAGAAAGACCTCC-3'
3' primer : 5'-CCCGGATCCCATGCTTCTAGAGGGCGG-3'
SIVmac239 Vpx
5' primer : 5'-CAGAGGACATATGTCAGATCCCAGGGA-3'
3' primer : 5'-CCCGGATCCTTATGCTAGTCCTGGAGG-3'
SIVagm Vpx
5' primer : 5'-GGTCTAGACATATGGCCTCAGGAGGGTGG-3'
3' primer : 5'-AAAGGATCCTCACAGGCCTGGAGCGG-3'
hIFN- γ cDNA
5' primer : 5'-TTAAAGCTTCCACCATGAAATATACAAGTTATAT CTTGGC-3'
3' primer : 5'-TTGGATCCTTACTGGGATGCTCTTCG-3'

Table 2. The percentages of apoptosis induced by cycloheximide, or HIV-1 Vpr and determined by Hoechst stain, P.I. Stain and TUNEL Stain in HeLa cells.

	Hoechst Stain	P.I. Stain	TUNEL Stain
Cycloheximide(0 µg/ml)	0.0%	1.5%	0.7%
Cycloheximide(100 µg/ml)	13.3±1.2%†	15.1%	17.6%
Empty vector	2.2±0.6%†	61.8%	55.5%
VSV-G	3.2±1.3%†	66.8%	47.7%
HIV-1 Vpr	9.6±1.2%†	55.6%	40.2%

HeLa cells were treated with 100 µg/ml cycloheximide or transfected with 5 µg tet-hGH-derived expression plasmids as well as 2 µg UHD-15. The percentages of apoptotic cells determined at 48 hr after treatment by three different apoptosis assay were listed. In Hoechst stain, quantitative analysis was performed by counting 500 cells in each examination. († n=3)

Table 3. The kinetics of cytotoxicity induced by HIV-1 Vpr or hIFN- γ in TE671 cells, determined by the MTT assay and the trypan blue exclusion method.

	24 hr		36 hr		48 hr	
	Trypan blue	MTT	Trypan blue	MTT	Trypan blue	MTT
Empty vector	30.0 $\pm$ 2.9%*	57.0 $\pm$ 2.2%*	51.0 $\pm$ 1.9%	48.0 $\pm$ 4.3%	40.7 $\pm$ 5.2%	43.5 $\pm$ 3.9%
HIV-1 Vpr	28.5 $\pm$ 5.2%*	48.3 $\pm$ 3.7%*	34.1 $\pm$ 1.9%	32.8 $\pm$ 2.8%	20.1 $\pm$ 4.5%	26.7 $\pm$ 3.1%
hIFN- γ	20.3 $\pm$ 4.9%*	44.7 $\pm$ 2.9%*	30.4 $\pm$ 3.3%	28.2 $\pm$ 4.1%	16.8 $\pm$ 3.7%	18.1 $\pm$ 3.4%

Cells were transfected with 5 μ g tet-hGH-derived expression plasmids as well as 2 μ g UHD-15. * means the viability of cells determined by the MTT assay or trypan blue exclusion method is significantly different from each other ($p < 0.05$, t test, $n = 6$).

Table 4. The percentage of apoptosis induced by additional HIV-1 Vpr and hIFN- γ plasmid constructs in various cultured cells

	HeLa	TE671	293	PTISK	Vero	CHTG49	P388D1	A9
Empty vector	n.d.	3.0 $\pm$ 0.5%	1.8 $\pm$ 0.4%	<1%	<1%	<1%	<1%	0%
VSV-G	2.3 $\pm$ 0.2%	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
hIFN- γ	8.5 $\pm$ 1.1%*	12.4 $\pm$ 1.2%*	6.4 $\pm$ 0.8%*	<1%	<1%	<1%	<1%	0%
HIV-1 Vpr	6.5 $\pm$ 0.8%*	13.0 $\pm$ 1.0%*	5.6 $\pm$ 0.6%*	<1%	<1%	<1%	<1%	0%

Cells were transfected with 5 μ g tat-hGH-derived expression plasmids as well as 2 μ g UHD 15-1 and the percentage of apoptotic cells was determined 48 hr (72 hr in 293 cells) after transfection. *, the percentage of apoptosis is significantly different from that of negative control e.g. VSV-G or empty vector ($p < 0.05$, t test, $n = 6$). The transfection of each construct was repeated three times in PTISK, Vero, CHTG49, P388D1, and A9 cells and six times in HeLa, TE671, and 293 cells. Quantitative analysis was performed by counting 500 cells in each examination. n.d., not determined.

Table 5a. The kinetics of apoptosis induced by cycloheximide at 24 and 48 hr in TE671 cells, determined by Hoechst stain or ^3H -thymidine release assay.

	Hoechst Stain		^3H -thymidine release	
	24hr	48hr	24hr	48hr
0 $\mu\text{g/ml}$	0.0%	0.0%	0.0%	0.0%
10 $\mu\text{g/ml}$	0.6%	4.4%	0.0%	0.8%
100 $\mu\text{g/ml}$	3.0%	12.8%	0.1%	2.6%

Table 5b. The amounts of ^3H -thymidine released into the supernatants of the non-transfected TE671 cells, induced by coculturing with HIV-1 Nef, HIV-1 Vpr, or hIFN- γ -transfected TE671 cells at different time points (n=5).

CPM	24hr	36hr	48hr	60hr	72hr
Spontaneous	1189 $\pm$ 100	2280 $\pm$ 197	3065 $\pm$ 199	5065 $\pm$ 213	7412 $\pm$ 301
HIV-1 Nef	1021 $\pm$ 135	2287 $\pm$ 312	2982 $\pm$ 170	5163 $\pm$ 115	7245 $\pm$ 498
HIV-1 Vpr	1065 $\pm$ 199	2379 $\pm$ 226	3133 $\pm$ 65	4918 $\pm$ 182	7384 $\pm$ 259
hIFN- γ	1105 $\pm$ 84	2201 $\pm$ 132	2902 $\pm$ 210	4832 $\pm$ 355	7512 $\pm$ 182
Total amounts of ^3H -thymidine absorbed by cells					369655 $\pm$ 5686

Table 6. The apoptotic effects of Vpr, Vpx of different lentiviruses in different cultured cells

	HeLa	TE671	293	PTISK	Vero	CHTG49	P388D1	A9
Empty Vector	1.8±0.8%	4.2±0.8%	2.7±0.4%	<1%	1.0±0.1%	<1%	<1%	0%
HIV-1 Nef	2.5±1.1%	5.1±1.3%	3.1±0.7%	<1%	1.7±0.1%	<1%	<1%	0%
hIFN- γ	9.7±1.6%*	16.7±2.7%*	8.7±1.2%*	<1%	1.5±0.3%	<1%	<1%	0%
HIV-1 Vpr	8.4±1.2%*	18.9±1.9%*	9.5±1.8%*	<1%	0.7±0.4%	<1%	<1%	0%
HIV-2 Vpr	1.3±0.5%	3.9±2.1%	3.0±0.6%	<1%	1.2±0.2%	<1%	<1%	0%
HIV-2 Vpx	2.1±0.9%	5.5±0.8%	2.8±1.0%	<1%	0.9±0.1%	<1%	<1%	0%
HIV-2 Vpr+Vpx	1.9±0.3%	4.7±1.3%	3.5±1.1%	<1%	1.1±0.3%	<1%	<1%	0%
SIV _{mac239} Vpr	1.7±0.4%	3.3±2.1%	3.3±0.6%	<1%	1.3±0.1%	<1%	<1%	0%
SIV _{mac239} Vpx	2.3±1.0%	3.9±1.4%	2.1±1.2%	<1%	0.8±0.1%	<1%	<1%	0%
SIV _{mac239} Vpr+Vpx	1.6±0.5%	4.2±0.7%	2.5±0.7%	<1%	1.2±0.2%	<1%	<1%	0%
SIV _{agm} Vpx	2.3±0.8%	5.7±0.4%	3.7±1.0%	<1%	0.8±0.2%	<1%	<1%	0%

Cells were cotransfected with 5 μ g pHEF1 α -derived expression plasmids and 0.1 μ g XGH5 plasmid and the percentage of apoptotic cells was determined at 48 hr (72 hr in 293 cells) after transfection. '*' means the percentage of apoptosis is significantly different from that induced by negative control e.g. HIV-1 Nef or empty vector ($p < 0.05$, 1 test, $n = 6$). The transfection of each construct was repeated three times in PTISK, Vero, CHTG49, P388D1, and A9 cells and six times in HeLa, TE671, and 293 cells. Quantitative analysis was performed by counting 500 cells in each examination. The transfection efficiency was checked routinely by measuring the amount of human growth hormone in the culture media.

Chapter V.

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